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Development of a technology for biomonitoring atmospheric deposition of toxic organic compounds and evaluation of their impact on ecosystem and human health

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RESUMO

A monitorização no ambiente de poluentes orgânicos persistentes (POPs), como os PCDD/Fs e PAHs, é urgente, pois estes são considerados substâncias tóxicas, cujos efeitos na saúde pública e nos ecossistemas podem provocar situações irremediáveis num futuro a médio prazo. Em geral, a monitorização é feita pontualmente no tempo e espaço, não permitindo a identificação de fontes poluidoras nem a análise de risco ao nível do território. O número de estações de monitorização é reduzido e as medições efectuadas são pontuais no tempo e/ou não se fazem rotineiramente (caso dos PCDD/Fs). A utilização de biomonitores para efectuar estas medidas tem vantagens, uma vez que alguns organismos biológicos possuem a capacidade de acumular os poluentes, fornecendo uma medida integrada da exposição num determinado período de tempo, o que pode ser mais relevante em termos de saúde pública (poluição crónica).

A utilização de biomonitores em estudos de poluição tem vantagens quando as concentrações no meio a testar estão abaixo dos limites de detecção e quando há necessidade de periodicidades de amostragem muito elevadas e a monitorização físico-química é difícil de implementar. A grande vantagem reside no facto das redes de amostragem de biomonitores serem operadas de um modo flexível e descentralizado. A utilização de biomonitores permite diminuir os encargos de instalação, manutenção e operação normalmente associados às estações tradicionais. Os biomonitores, sendo organismos vivos que reagem diferenciadamente à poluição, permitem ainda avaliar o impacto dos poluentes ao nível do ecossistema.

Segundo a União Europeia, as concentrações no ar e as medidas de deposição em biomonitores são consideradas como sendo as mais indicadoras para monitorizar o impacto das medidas de restrição das emissões atmosféricas de compostos orgânicos. Entre os biomonitores de compostos orgânicos mais utilizados, destacam-se vegetais, musgos, leite materno, peixes, bivalves, frangos, agulhas de pinheiro e líquenes. A biomonitorização através de líquenes é já um processo implementado a nível legal em vários países europeus, devido à eficiência e baixo custo/benefício associados. Têm igualmente sido efectuadas medições de compostos orgânicos no solo, sedimentos e no ar. O solo funciona como sink para os compostos orgânicos, podendo por vezes atingir concentrações muito elevadas.

As publicações visando a utilização de biomonitores de compostos orgânicos não avaliaram a influência dos factores climáticos, nem efectuaram calibrações entre as concentrações medidas no biomonitor e as concentrações medidas no ar, no solo e na água por métodos físico-químicos. Este tipo de calibração é importante, pois permite a regulamentação da metodologia de biomonitorização, como complemento das medições físico-químicas.

O principal objectivo deste trabalho consiste em desenvolver uma tecnologia para biomonitorizar poluentes orgânicos persistentes e avaliar o seu impacto ao nível do ecossistema e da saúde pública. Os biomonitores que servirão de base a este estudo serão os líquenes e musgos aquáticos, por serem considerados biomonitores ideais na monitorização dos mais variados poluentes.

Ao longo desta tese, procurar-se-á estudar os factores que influenciam a intercepção e acumulação de POPs pelos líquenes, mostrar como os líquenes e musgos aquáticos podem ser usados para identificar diferentes fontes de poluição por POPs em ambientes terrestres e aquáticos, e exemplificar como os biomonitores podem contribuir para estudos de saúde humana de carácter ambiental. Este trabalho integra diferentes tipos de conhecimento, permitindo desenvolver uma tecnologia integrada de biomonitorização.

Para isso, o trabalho foi dividido em cinco capítulos. No primeiro capítulo apresenta-se uma introdução geral sobre o tema, focada no actual estado da arte, e nas limitações dos métodos de monitorização ambiental, que justificam a necessidade de desenvolver o presente estudo. Para além de toda uma informação relativa a POPs, apresenta-se uma descrição dos trabalhos desenvolvidos nos últimos anos usando líquenes e musgos aquáticos como biomonitores de toda uma panóplia de poluentes, de forma a explanar a necessidade de estudar um conjunto de factores que podem influenciar a acumulação de POPs por estes organismos.

No segundo capítulo, o objectivo consiste em optimizar a uso de líquenes como biomonitores de POPs. Apesar dos líquenes terem sido usados ao longo de décadas como biomonitores de metais e de outros poluentes, até à data apenas alguns estudos foram desenvolvidos usando líquenes para monitorizar POPs. Desta forma, revelava-se necessário estudar os factores que contribuem para a intercepção e acumulação de PCDD/Fs e PAHs nos líquenes. Questões relacionadas com a influência da forma de

crescimento, tamanho e idade dos líquenes, assim como a influência de aspectos metodológicos (tais como a influência do substrato) na performance destes organismos enquanto biomonitores de POPs serão analisadas (capítulo 2.1). Neste segundo capítulo, os líquenes serão comparados com outros métodos de monitorização, tais como agulhas de pinheiro, ar e solo (capítulos 2.2 e 2.3). De forma a poder transformar as concentrações de POPs medidas nos líquenes em valores equivalentes reconhecidos pela legislação, serão efectuadas calibrações entre os líquenes e amostras de ar e de solo (capítulos 2.3 e 2.4).

Um dos principais desafios dos estudos de monitorização ambiental é rastrear fontes de poluição. Isto pode ser uma tarefa complicada em ambientes onde coexistem diferentes tipos de indústria, áreas urbanas, actividades agrícolas, etc., todas elas contribuindo para o *input* de POPs no ambiente. O objectivo do terceiro capítulo é mostrar como os líquenes e musgos aquáticos podem ser usados para identificar diferentes fontes de poluição em meio terrestre (capítulo 3.1) e em meio aquático (capítulo 3.2). Para tal, será apresentada uma análise integrada de diferentes níveis de informação. Numa mesma abordagem serão integradas informações relativas a concentrações de POPs e metais em biomonitores, rácios entre diferentes compostos de POPs, e uso do solo, de forma a identificar a origem da poluição por POPs.

No quarto capítulo será mostrado como a biomonitorização com líquenes pode ser usada como complemento a estudos de saúde pública (capítulos 4.1 e 4.2). Em estudos de saúde pública, quando o objectivo consiste em relacionar poluição com saúde humana, uma das principais dificuldades é avaliar que populações devem ser consideradas como controlo e que populações devem ser consideradas como estando expostas aos poluentes. Esta limitação é consequência da falta de resolução espacial dos dados de deposição de poluentes. Normalmente, os dados de uma estação de monitorização da qualidade do ar são considerados como sendo representativos de toda uma região, e como tal, o nível de exposição humana aos poluentes é considerada como sendo igual em toda a área. A utilização de biomonitores ambientais pode ser útil, uma vez que permite obter dados de poluição com uma elevada resolução espacial. No caso particular dos POPs, usar os líquenes como acumuladores de longo-termo, permitirá obter informação sobre a exposição crónica a estes compostos via inalação (capítulo 4.2).

Finalmente, o capítulo 5 consiste numa discussão geral, onde todo o conhecimento adquirido durante os capítulos anteriores será integrado e interpretado, de forma a delinear um conjunto de directrizes e procedimentos a seguir na biomonitorização de poluentes orgânicos persistentes, usando líquenes e musgos aquáticos.

PALAVRAS-CHAVE: PCDD/Fs, PAHs, líquenes, musgos, exposição humana

ABSTRACT

During the last decades, awareness regarding persistent organic pollutants (POPs), such dioxins and furans (PCDD/Fs) and polycyclic aromatic hydrocarbons (PAHs), has become a cutting-edge topic. Features such as toxicity, bioaccumulation and persistence of these compounds in the environment, contributed for their inclusion in the Convention on Long-Range Transboundary Air Pollution Protocol (LRTAP) and on the Stockholm Convention as pollutants for which emissions must be reduced. Monitoring of PCDD/Fs and PAHs in air and water has proven to be an insufficient method to capture the real picture of dispersion and deposition of these compounds; to overcome this limitation, environmental biomonitoring using lichens and aquatic bryophytes, have aroused as promising tools. Though many studies have been performed using these organisms as biomonitors of a wide range of pollutants (such as heavy metals, radionuclides, gaseous pollutants, etc.), their use as POP biomonitors is still in a germinal stage and needs further study. The main aim of this thesis is study the factors that influence the interception and accumulation of POPs by lichens, how lichens and aquatic bryophytes can be used to track different pollution sources, and how can these biomonitors contribute to environmental health studies. This work will integrate different types of knowledge; allow developing an integrated biomonitor technology to be used to assess environmental POP pollution.

KEYWORDS: PCDD/Fs, PAHs, lichens, mosses, human exposure

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LIST OF ABBREVIATIONS

ACPH	acenaphtene
ACPHY	acenaphtylene
ANTH	anthracene
BaA	benzo[a]anthracene
BaP	Benzo[a]pyrene
BaPeq	benzo[a]pyrene equivalents
BbFA	benzo[b]fluoranthene
BghiP	benzo[g,h,i]perylene
BkFA	benzo[k]fluoranthene
CHR	chrysene
DBahA	dibenzo[a,h]anthracene
EF	enrichment factors
FA	fluoranthene
FLU	fluorene
HMW-PAHs	High molecular weight PAHs
HpCDD	heptachlorodibenzo-*p*-dioxins
HpCDF	heptachlorodibenzofuran
HxCDD	hexachlorodibenzo-*p*-dioxins
HxCDF	hexachlorodibenzofuran
IP	indeno[1,2,3-cd]pyrene
I-TEQ	International toxic equivalents
Kow	partition coefficient octanol-water
LMW-PAHs	Low molecular weight PAHs
NAPH	naphthalene
OCDD	octachlorodibenzo-*p*-dioxin
OCDF	octachlorodibenzofuran
PAHs	polycyclic aromatic hydrocarbons
PCDD/PCDFs	dioxins and furans
PCDDs	polychlorinated dibenzo-*p*-dioxins
PCDFs	polychlorinated dibenzofurans
PeCDD	pentachlorodibenzo-*p*-dioxins
PeCDF	pentachlorodibenzofuran
PHEN	phenanthrene
POPs	persistent organic pollutants
PY	pyrene
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCDF	2,3,7,8-tetrachlorodibenzofuran
TEFs	toxic equivalent factors
TEQs	toxicity equivalents
TSP	total suspended particles

Chapter 01 | General introduction

Chapter 01 | General introduction

1.1. Environmental pollution and public health – *an overview*

Over the last decades a global concern over the public health impacts attributed to environmental pollution has been increasing. The World Health Organization (WHO) estimates that about a quarter of the diseases facing mankind today occur due to prolonged exposure to environmental pollution. The underlying causes of these environment-related diseases are however not easily identified and may be acquired along the life.

The concern about effects of pollution in human health is not recent. Humans have been exposed to anthropogenic sources of air pollution since they lit their first fire. The air of the earliest towns was rife with smoke and noxious odours emanating from trades such as tanning. However, only with the general use of coal, did air pollution begin to be a major problem. Until the early Middle Ages, wood was the prime source of heat throughout Europe. The use of coal fouled the air so badly that in 1273 England's King Edward I passed a law prohibiting the burning of at least one type of coal, and in the early 1400s Henry V formed a commission to oversee the use of coal in London. In 1661, Charles II ordered scientist John Evelyn to survey the effects of the increasing air pollution over the city. Evelyn recognized the relationship between the "dismal cloud" over London and the number of fatal diseases, but his warnings about the need for air-pollution controls were ignored.

After the introduction of coal, build-ups of air pollution sporadically afflicted towns, but urban centres still had fairly small populations, industry operated on a small scale, and the outpourings of industrial contaminants were not yet the norm. Thus, the effects of early episodes of air pollution were relatively minor.

By the late 1800s, industry was booming, larger and larger populations were concentrating in cities, and increasing amounts of chemical pollution were entering the air. As a result, in December 1873, when particularly adverse weather conditions occurred, smog of pollution (mainly formed by SO_2 and soot, particles) gathered over London. This episode resulted in 1150 deaths, making it one of the earliest air-pollution disasters (Halliday, 1961). Since 1873, in the industrialized world there have been at

least other similar 40 episodes (Noji, 1997). Together with these gaseous pollutants, concerns about metal pollution have increased. One of the most known incidents was the one which occurred with mercury in Minamata, Japan. In 1953, 121 residents of the coastal region along the Minamata Bay became sick with paralysis and disturbances in their sight and hearing. Cases of human toxicity due to environmental exposure to heavy metals, such as Cd, Al, Pb, Ni, Cr, among others, have been reported over the years (Fellenberg, 2000).

More recently, a new kind of pollutants has started being of concern – persistent organic pollutants (POPs). Many POPs were widely used during the boom in industrial production after World War II, when thousands of synthetic chemicals were introduced into commercial use. Many of these chemicals proved beneficial in pest and disease control (like DDT, a well known pesticide), crop production, and industry. These same chemicals, however, have had unforeseen effects on human health and the environment.

DDT (dichlorodiphenyltrichloroethane) is likely one of the most famous and controversial pesticides ever made. The heavy use of this highly persistent chemical led to widespread environmental contamination and the accumulation of DDT in humans and wildlife - a phenomenon brought to public attention by Rachel Carson in her 1962 book, *Silent Spring*. A wealth of scientific laboratory and field data have now confirmed research from the 1960s that suggested, among other effects, that high levels of DDE (dichlorodiphenyldichloroethylene, a metabolite of DDT) in certain birds of prey caused their eggshells to thin so dramatically they could not produce live offspring. One bird species especially sensitive to DDE was the bald eagle. Public concern about the eagles' decline and the possibility of other long-term harmful effects of DDT exposure to both humans and wildlife prompted the Environmental Protection Agency (EPA) to cancel the registration of DDT in 1972. The bald eagle has since experienced one of the most dramatic species recoveries in our history. DDT was probably the first POP that generated a public awareness regarding its potentially toxic effects on human health.

In addition to these intentionally produced compounds for commercial use, POPs also include not intentionally produced compounds, such as dioxins and furans (PCDD/Fs) and polycyclic aromatic hydrocarbons (PAHs), which result from some industrial processes and from combustion.

One of the first health concerns about PCDD/Fs was about the use of the Agent Orange as defoliant agent during the Vietnam War. Agent Orange was the code name for an herbicide developed for the military, primarily for the use in tropical climates. The purpose of the product was to deny an enemy cover and concealment in dense terrain by defoliating trees and shrubbery where the enemy could hide. Agent Orange was essentially a 50-50 mix of 2,4-D (2,4-cichlorophenoxyacetic acid) and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid). The combined product was mixed with kerosene or diesel fuel and dispersed by aircraft, vehicle, and hand spraying. An estimated 19 million gallons of Agent Orange were used in South Vietnam during the war. The earliest health concerns about Agent Orange were about the products contamination with TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin, the most toxic PCDD/Fs). Numerous diseases (such as chloroacne, lymphomas, prostate cancer, respiratory cancers including cancers of lungs, larynx, trachea and bronchus, etc.) have been reported in Vietnam populations after exposure to the Agent Orange. However, the general public first became aware of PCDD/Fs when, in 1976, in Seveso, in the vicinity of Milan, Italy, TCDD escaped into the environment as a result of a failed synthesis at a chemical plant, causing the death of numerous animals in the surrounding area (Fellenberg, 2000). Due to persistency and lipophilic character of these compounds, they are retained during long periods in soil and sediments, suffer bioaccumulation in living organisms and enter into the food-chains of all animals, including humans. Even today, after decades of the use of Agent Orange and of the Seveso accident, high concentrations of PCDD/Fs are found in soils and in living organisms of those regions. Once introduced into the environment, either in air, soil or water, POPs will persist for long periods, potentially affecting the human health in the long-term. In this way, it's crucial to monitor these compounds in all environmental matrices, in order to be able to protect the ecosystem and human health.

1.2. Persistent Organic Pollutants (POPs)

POPs are organic chemical substances, carbon-based, which possess a particular combination of physical and chemical properties such that, once released into the environment, they: i) remain intact for exceptionally long periods of time (many years); ii) become widely distributed throughout the environment as a result of natural processes involving soil, water and, most notably, air; iii) accumulate in the fatty tissue of living organisms including humans, and are found at higher concentrations at higher

levels in the food chain; and iv) are toxic to both humans and wildlife (Jones and Voogt, 1999).

As a result of releases to the environment over the past several decades due especially to human activities, POPs are now widely distributed over large regions (including those where POPs have never been used) and, in some cases, they are found around the globe (Hung et al., 2010). This extensive contamination of environmental media and living organisms includes many foodstuffs and has resulted in the sustained exposure of many species, including humans, for periods of time that span generations, resulting in both acute and chronic toxic effects (UNEP, 2012).

With the evidence of long-range transport of these substances to regions where they have never been used or produced and the consequent threats they pose to the environment of the whole globe, the international community has now, at several occasions called for urgent global actions to reduce and eliminate releases of these chemicals.

An international action has therefore been taken in the form of two international agreements to protect human health and the environment from POPs: i) The 1998 Aarhus Protocol on Persistent Organic Pollutants to the Convention on Long-Range Transboundary Air Pollution (LRTAP, 1998), a treaty for the UNECE (United Nations Economic Commission for Europe region); and ii) The 2001 Stockholm Convention on Persistent Organic Pollutants, a global treaty under the United Nations Environment Programme (UNEP, 2001). Regulation (EC) No 850/2004 of the European Parliament on persistent organic pollutants and amending Directive 79/117/EEC1 implement both of these agreements for all of EU member states, aiming to eliminate and/or restrict the production and use of selected POPs.

1.3. PAHs and PCDD/Fs as unintentionally produced POPs

Polycyclic aromatic hydrocarbons (PAHs) and polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), in addition to hexachlorobutadiene, are included by the Convention on Long-Range Transboundary Air Pollution Protocol and Stockholm Conventions in the list of unintentionally produced POPs (by-products) whose emissions should be reduced. Unlike other POPs, these compounds have never been produced for technical or commercial use but are a side

product of not just waste combustion but several industrial processes (Kulkarni et al., 2008; Longwell, 1982; Haynes, 1991; Ravindra et al., 2008; Chagger et al., 2000). They are mainly formed as a consequence of incomplete combustion of organic matter. In the case of PAHs, these can also arise from unburned fuel and lubrificating oil (petrogenic sources, without combustion), as well as they can be formed during food processing and manufacturing (Figure 1).

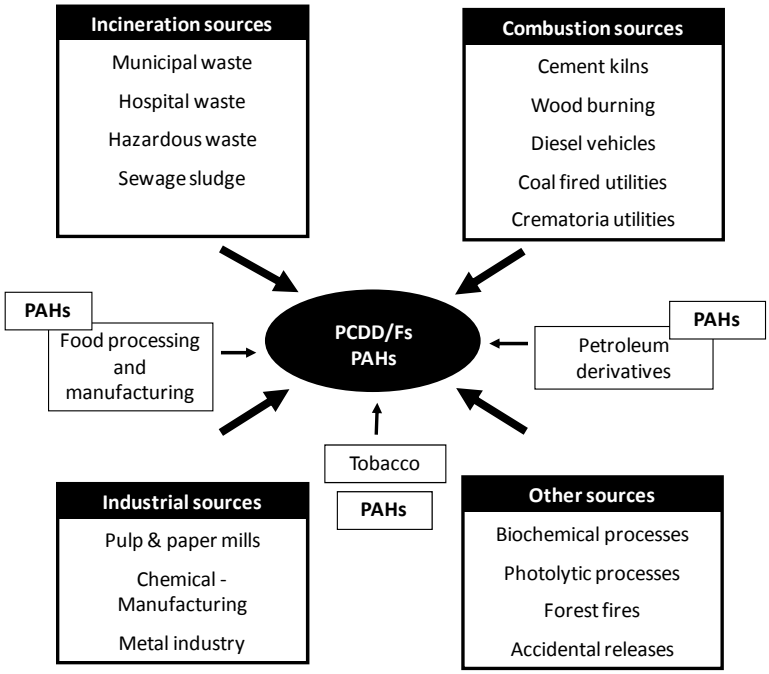

Figure 1. Sources of PCDD/Fs and PAHs. Adapted from Kulkarni et al, 2008.

Chemically, PAHs are persistent organic pollutants formed by two or more aromatic rings, made up of carbon and hydrogen atoms. The U.S. Environmental Protection Agency (EPA) has promulgated 16 unsubstituted PAHs (EPA-PAH) as priority pollutants to be monitored in the environment (EPA, 1986) (Figure 2). Ranging from 2- to 6-rings and with molecular weight from 128 to 278 g/mol, these compounds can be classified as low molecular weight PAHs (LMW-PAHs, with 2-3 rings) and high molecular weight PAHs (HMW-PAHs, with 5-6 rings). LMW-PAHs are usually emitted by petrogenic sources (without combustion), while HMW-PAHs are usually related to pyrogenic sources (with combustion) (Meador et al., 1995; Jones and Voogt, 1999).

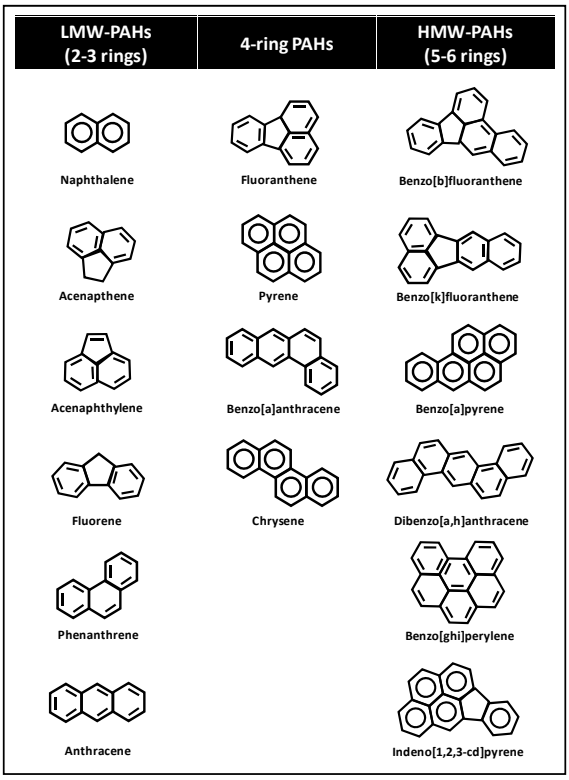

Figure 2. Chemical structures of the 16 EPA-PAHs.

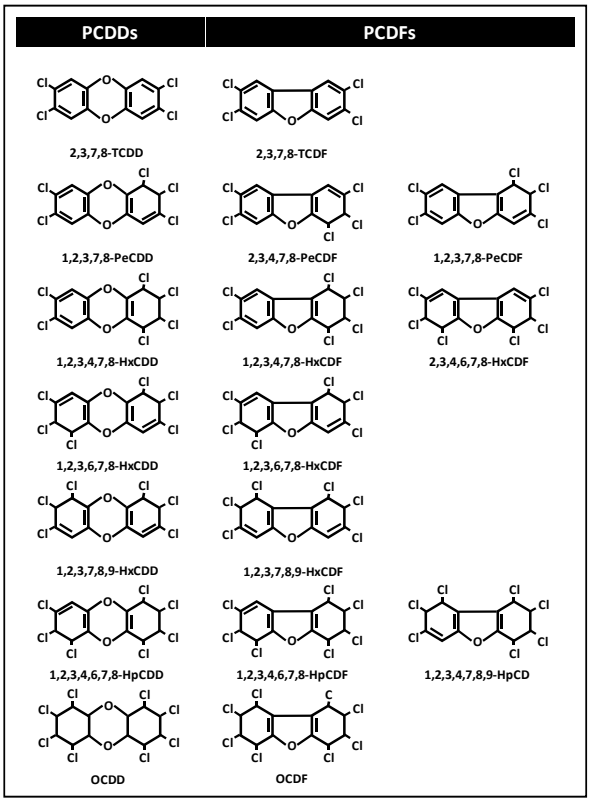

Figure 3. Chemical structures of the 17 toxic PCDD/F congeners.

PCDD/Fs are a group of more than 200 organic compounds, formed by two benzene rings joined by two (in the case of PCDDs) or one (in the case of PCDFs) oxygen bridges. PCDD/Fs can be divided according to their homologue groups (from TCDD/Fs, with four chlorine atoms to OCDD/Fs, with eight chlorine atoms), and to their congener groups (which vary with the position of chlorine atoms in the molecule). The toxicity of PCDD/Fs is related with the presence of at least 4 chlorine atoms in the positions 2, 3, 7 and 8 of the molecule; and thus there are 17 toxic congeners (Figure 3) (Lohmann and Jones, 1988).

Homologue and congener profiles, that is the relative contribution of each homologue group or each toxic congener to the total PCDD/F content, have been used as tracers of numerous pollution sources and environmental fate of these compounds (Alcock et al., 2001; Lohmann and Jones, 1988).

1.4. Human exposure to POPs

Both, PAHs and PCDD/Fs, are toxic organic compounds, whose impact on human health is mainly related with carcinogenic, mutagenic and teratogenic effects. Several compounds of these groups have been classified by the International Agency for Research on Cancer (IARC) as carcinogenic (Group 1) or probable (2A) or possible (2B) human carcinogens (IARC, 1987).

Studies have shown that PCDD/Fs and PAHs interact with receptors, such as the hormonal receptors; the most studied one has been the aryl hydrocarbon receptor (AhR). Environmental contaminants, such as the PAHs and PCDD/Fs (namely TCDD), represent the most extensively characterized classes of AhR ligands (Denison et al, 1998; Denison and Heath-Pagliuso, 1998; Poland and Knutson, 1982; Safe, 1990), although naturally occurring ligands do exist. The presence of the AhR and AhR signal transduction pathways in a diverse range of species, tissues, and cell types (Bank et al., 1992; Holmes and Pollenz, 1997; Hahn, 1988), combined with its ability to act as a ligand-dependent transcription factor, suggests that many of the toxic and biological effects of AhR ligands (such as PAHs and PCDD/Fs) result from differential alteration of gene expression in susceptible cells. Because many of the adverse effects of PCDD/Fs and PAHs are not observed until days to weeks following chemical exposure (Poland and Knutson, 1982; Devito and Birnbaum, 1994), the adverse effects of these chemicals likely result from the continuous and inappropriate expression of specific genes in

responsive cells. Though there are cases of acute exposure to PCDD/Fs and PAHs, the biggest issue is the chronic exposure to low levels of these compounds (Rappe 1993, WHO 1992, Fiedler et al. 1990). Due to their lipophilic nature, PCDD/Fs and PAHs amplify and bioaccumulate in living organisms, entering into the foodweb of all animals, including humans, triggering harmful effects on health (Rappe 1993, WHO 1992, Fiedler et al. 1990).

Humans can be exposed to POPs through diet, occupation, accidents and both the indoor and outdoor environments. Human exposure can occur by various pathways, notably inhalation of air and resuspended soil particles, ingestion of food (which may include maternal milk), water and soil particles (relevant in the case of children), and dermal contact to soil and water (USEPA, 1998) (Figure 4). Exposure can either be a short-term exposure to high concentrations (acute) or long-term exposure to lower concentrations (chronic). Chronic exposure occurs most commonly via dietary exposure pathways, though the contribution of other pathways should not be neglected. Contamination of food may occur through environmental pollution of the air, water and soil, or through the previous use or unauthorized use of organochloride pesticides on food crops. Foods containing the greatest concentrations of POPs include the fatty tissues of animals and edible oils. The contamination of food, including breast milk, by POPs is of worldwide concern (Stober, 1998).

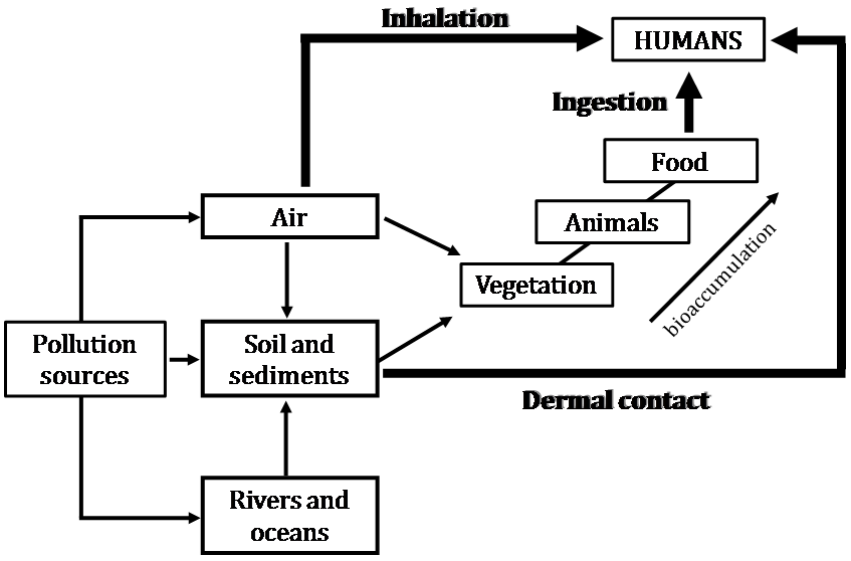

Figure 4. Human exposure pathways to POPs.

To reliably assess human exposure to environmental POPs, concentrations of POPs in different environmental compartments (air, soil, water) need to be accounted. Usually these concentrations are obtained through:

i) Estimation of levels deposited in air, soil and water, based on POP emission data from known pollution sources (industrial sources); however, the uncertainty associated with the places where pollutants are predicted to deposit and where they actually are being deposited is very high, increasing also the uncertainty of the exposure data.

ii) Measurements of POPs performed in air monitoring quality stations; these stations, normally only few in space, tend to be located at specific sites selected for their expected relatively high or low concentrations and are often placed at a much higher altitude than the human breathing zone (EHC 27, 1983); moreover, the measurements reflect a short-term indicator that varies in time and which does not reflect the levels that populations are exposed in space and in the long-term.

iii) Measurements of POPs in soil and water samples.

These lead to a lack of spatial resolution in environmental epidemiological studies, as populations living in a large region are considered to be exposed to the same level of pollutants. One of the major challenges in environmental health studies is to assess which populations can be considered as control and which ones can be considered as exposed. In this way, there is a need to develop a tool that allows assessing human exposure to environmental POPs in the long-term and with a spatially explicit scale.

1.5. Toxicity Equivalent Factors (TEFs)

PCDD/Fs and PAHs exist in environmental and biological samples as complex mixtures of various compounds whose relative concentrations differ across trophic levels. These differences are caused by environmental degradation, which is dependent on compounds' features, such as solubilities, volatilities, and rates of degradation/metabolism. As a result, these mixtures change spatially and temporally into the environment and are very different from the technical mixtures originally released into the environment (Jones and Voogt, 1999).

The complex nature of PCDD/Fs and PAHs mixtures complicates the risk evaluation for humans and wildlife. For this purpose, the concept of toxic equivalency factors (TEFs) has been developed and introduced to facilitate risk assessment and regulatory control of exposure to these mixtures (USEPA, 2010). The TEF concept considers the relative toxicity of each compound in the mixture in relation to the most toxic compound – TCDD, in the case of PCDD/Fs and benzo[a]pyrene in the case of PAHs – which are the ones that have proven to be carcinogens to humans. The overall toxicity of the mixture is achieved, using equation 1, as follows:

$$TEQ = \sum_{i=1}^{n}(Ci \times TEFi) \text{ (Equation 1)}$$

The relative toxicity of each compound is determined on the basis of available *in vivo* and *in vitro* data. However, it should also be understood that the TEF concept is based on a number of assumptions and has limitations. In this respect, the most basic assumption is that the combined effects of the different compounds are dose or concentration additive. Other assumptions are: the AhR receptor mediates most if not all of the biologic and toxic effects of PCDD/Fs and/or PAHs; the applicability of extrapolations from short-term bioassays to long-term health effects; similarities between interspecies kinetics and potency; appropriateness of high-dose to low-dose extrapolations; and the constancy of TEF relationships for different exposure routes, health endpoints, and dose levels (USEPA, 1989, 2000, 2003; Birnbaum and DeVito, 1995; Birnbaum, 1999). Due to the everyday increasing of studies regarding toxic effects of both PCDD/Fs and PAHs, TEFs have been subjected to revisions over the years (USEPA, 2010).

1.6. Factors affecting transport, deposition and fate of POPs

Once emitted to the atmosphere by pollutant sources, the transport, deposition, and fate of POPs in the environment are dependent on the physicochemical properties of the compounds. The distance that a compound is able to travel is dependent on its atmospheric lifetime, phase (gas or particle) in which it exists in the atmosphere and regional and global wind patterns (Jones and Voogt, 1999).

POP deposition from the atmosphere depends on the affinity for that compound to partition from the atmosphere to other environmental compartments. The mechanisms by which POPs undergo deposition are dependent on the phase of the compound in the atmosphere and the physicochemical properties that describe the affinity for the

compound to partition from the atmosphere to a different environmental compartment (Finizio et al., 1997; Simonich and Hites, 1995; Daly and Wania, 2005; Wania and Mackay, 1993).

POPs can undergo deposition through wet and/or dry processes and through air-surface exchange (Buckley, 1982; Welsch-Pausch et al., 1995; LeNoir et al., 1999). Wet deposition is the washout of gas and particle phase POPs in the atmosphere via precipitation events, while dry deposition is the gravitational fall out of higher mass particles.

Once deposited, the environmental fate of POPs is dependent on the affinity of the POP for the atmosphere compared to lipid-based matrices, such as vegetation in terrestrial environments and fish in aquatic environments. The octanol-water partition coefficient, K_{OW}, has been used to describe the accumulation potential of POPs in aquatic ecosystems (Gobas and MacLean, 2003). Some POPs have a high affinity for lipophilic matrices relative to water; therefore there is strong evidence of bioaccumulation of some POPs in aquatic organisms (Kelly, 2007). This K_{OW} coefficient has also been used as a measure of the lipophilic, non soluble, character of a compound; as thus as a measure of the potential bioaccumulation in terrestrial living organisms.

1.7. Environmental monitoring - conventional methods

After being released by pollution sources (known and unknown), POPs will disperse in the atmosphere and deposit on ecosystems (soil, water, vegetation) where they will bioaccumulate, contaminating the human and other animal food-chains (Figure 5).

Environmental monitoring of POPs can be performed using different methods. The most used one, which is required by the actual regulations, is to measure POPs in emissions from industries and estimate the dispersion and deposition based on these data; the main limitation of this method is that only major industries perform these measurements, and thus a set of small industries and also unknown/unexpected pollution sources are not accounted. As a consequence, most of the times the deposition models built in this way do not reflect what is actually being deposited. These measurements are useful to control emissions from industries, and not properly to assess deposition on ecosystems and human exposure. In this way, monitoring concentrations of POPs in environmental samples, such as air, water and soil is an

indispensable task, in order to improve information regarding POP deposition and fate in ecosystems.

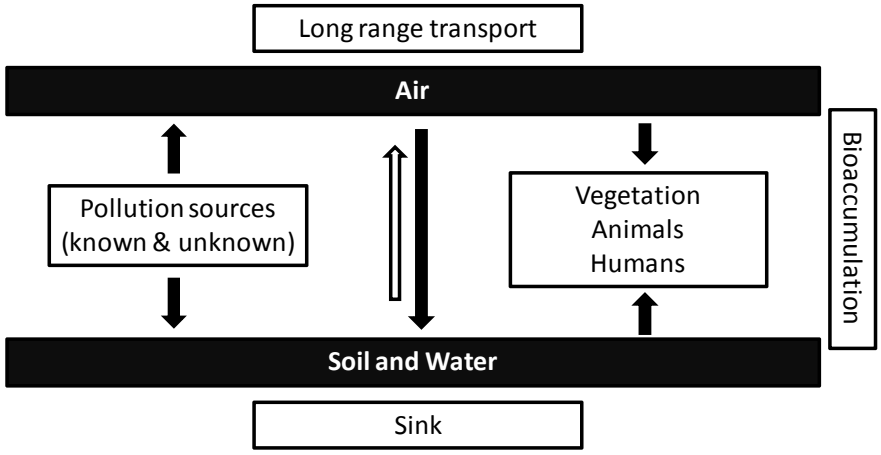

Figure 5. Environmental fate of POPs.

To determine POP concentrations in air, several sampling devices have been used. Active high volume air samplers (HiVols), powered by electricity, are most frequently used because samples are obtained over short periods of time (~24 hours), collect large volumes of air (600-800m3), and allow for the collection of POPs during episodic transport events (Bailey et al., 2000; Killin et al., 2002; Harner et al., 2005). These samplers use a continuous duty blower to suck in an air stream. When fitted with a particle size classifier, it separates particles (usually greater than 10μm size) from the air stream. The air stream is then passed through a filter paper to collect particles lesser than 10μm size (PM10). Gravimetric measurements yield values of suspended particulate matter (SPM), as the sum of the two fractions, and PM10, the material retained on the filter paper. The filter paper can be used to determine POPs in the particle-phase of air. The sampler can also be used to sample POPs in the vapour-phase or air. For that, a stream of unfiltered air passes through polyurethane foam (PUF) embedded in a solvent (such as acetonitrile) after passing through the filter; the foam will retain the vapour-phase compounds (Ockenden et al., 1998, 2001; Gouin et al., 2005; Shoeib and Harner, 2001). As POPs exist at low concentrations in air, most of the times they're not detectable using this method. In addition, this method requires the installation of expensive equipment, and thus generally a single monitoring station is

used to obtain information for large areas. For example, in Portugal, monitoring stations measuring PCDD/Fs in air are inexistent, and only a few (four) started recently measuring PAHs (APA, 2012).

Regarding POPs in water, measurements are performed through water analysis. For that, water samples are collected, concentrated and analysed. Such as in air, POPs in water exist at low concentrations, and thus most of the times are not detectable. Several studies have shown that POPs tend to disperse and deposit on sediments, after being released to water (Meador et al., 1995). Moreover, due to their lipophilic character and hydrophobicity, PAHs and PCDD/Fs are easily captured and bioaccumulated by living organisms. This makes the detection of illegal discharges into water bodies a difficult task. Finally, regarding POPs in soils samples, this matrix usually has high levels of POPs, as it's a natural sink for persistent and lipophilic compounds, such as PAHs and PCDD/Fs, which absorb to soil organic carbon and, once absorbed remain relatively immobile (Fiedler, 1999). Soil is considered a typical accumulating matrix with a long-term memory, and thus it doesn't respond quickly to changes in emissions (Fiedler, 1999). Moreover, it tends to be more concentrated in high molecular PAHs and in high chlorinated PCDD/Fs, which are more stable than the lighter compounds (Fiedler, 1999). Accumulation of POPs in soils may lead to further potential accumulation of vegetables (through ressuspension of soil particles) and food-chains, and then cause direct or indirect exposure to humans.

1.8. Using biomonitors to fulfil the gaps

The term biomonitor is used to refer to an organism, or a part of it, that depicts the occurrence of pollutants on the basis of specific symptoms, reactions, morphological changes or concentrations (Markert et al. 1997). To be considered a good biomonitor of air pollution, the organism should meet the following requirements (Wittig 1993, Conti & Cecchetti 2001; Sloof, 1993): i) accumulate detectable concentrations of pollutants; ii) be widely distributed and abundant; iii) be available during the whole year, not showing seasonal variations, and be easy to collect; iv) uptake and accumulate pollutants in a way that relates with the exposure period and intensity of pollution. Lichens and bryophytes are considered to be the best for use as biomonitors of air pollutants (Rühling & Tyler 1968, Puckett 1988).

Lichens are symbiotic organisms that have been used during decades as biomonitors of air pollution (Martin & Coughtrey, 1982; Puckett, 1988; Nimis et al, 1990). In fact, these organisms are among the most used in terrestrial environments for monitoring purposes. Lichens are symbioses between a fungus (mycobiont partner) and algae and/or cyanobacteria (photobiont partner). Both, mycobiont and photobiont form an integrated lichen thallus, which can be organized in either a stratified or non stratified way (Büdel and Scheidegger, 1996). Lichens with a non stratified arrangement are composed of a simple and undifferentiated thallus with irregularly distributed algae (Büdel and Scheidegger, 1996); whereas in stratified lichens, algae are confined to a distinct layer, and there is, at least, one more layer - the medulla - and other layer- the cortex – in which algae are not present (Jahns, 1973; Büdel and Scheidegger, 1996). The medulla consists of loosely interwoven hyphae with a very high internal air space and occupies the major part of the internal volume of the thallus (Jahns, 1973; Büdel and Scheidegger, 1996). Most lichens are protected against high irradiance and ultra-violet light by a thin to thick cortical layer, composed of more or less compressed hyphae (Hale, 1974; Büdel and Scheidegger, 1996; Solhaug et al., 2003). A lower cortex can also be present and is similar to the upper cortex in thickness and structure (Hale, 1974; Büdel and Scheidegger, 1996). In lichens where the lower cortex does not exist, the medulla corresponds to the lower layer of the thallus (Büdel and Scheidegger, 1996). Lichens can be generally grouped into three growth forms: crustose, foliose and fruticose (Hale, 1974; Büdel and Scheidegger, 1996). Crustose lichens don't have a stratified arrangement and don't possess a lower cortex and are tightly attached to the substrate by the hyphae of the medulla, being very difficult to separate them from it; foliose lichens are leaf-like, with flattened lobes, dorsiventral thallus and are attached to the substrate by rhizines or rhizoidal hyphae; fruticose lichens are hair-like, strap-shaped or shrubby, with radial or dorsiventral thallus and are, in general, attached to the substrate by a holdfast (Jahns, 1973; Hale, 1974; Büdel and Scheidegger, 1996).

Several features contribute for the success of lichens as biomonitors, notably: i) they don't have root neither a cuticle (lipid surface layer that is present in plants), meaning that the unique pathway for their nutrition is through atmospheric deposition (either of nutrients or pollutants); ii) they have a constant morphology throughout the year allowing collecting/studying them at any season; iii) they're long-lived organisms, being able to accumulate pollutants over their lifetimes; iv) they're widely distributed over the

planet, being possible to find them in almost all kind of environments (Manning & Feder, 1980; Martin & Coughtrey, 1982; Puckett, 1988; Garty, 1993; Sloof, 1993).

Due to these features, they've been extensively used to biomonitor pollution by heavy metals (Pb, Cu, Cr, Hg, Cd, Zn, As, Co, Mn, Ni), dust (Ca, Mg, K, Si), radionuclides (137Cs, 54Mn, 95Zr, 95Nb, 140Ba, 140La), gaseous pollutants (SO2, HF, NOx, NH4+, O3), anions (Cl-, NO3-), and more recently organic compounds (PCBs, PAHs, HCBs, PCDD/Fs). They have been successfully used to monitor pollution from road traffic, cities, industries, mines, the use of fertilizers in agriculture, volcanoes, etc. (Branquinho et al., 2008; Branquinho, 1997; Manning & Feder, 1980; Martin & Coughtrey, 1982; Puckett, 1988; Garty, 1993).

Lichens obtain their nutrients from wet and dry deposition (Martin and Coughtrey 1982, Garty 1993). The accumulation of pollutants occurs through a number of different mechanisms: as layers of particles or entrapment on the surface of the cells, incorporation into the outer walls of the cells through ion exchange processes, and metabolically controlled passage into the cells (Branquinho et al., 1999; Branquinho, 2001; Brown, 1991; Brown and Bates 1990; Tyler 1990; Puckett, 1988; Brown, 1991; Garty, 1993; Richardson, 1995). The attachment of particles is affected e.g., by the size of the particles and the surface structure of the lichens. Ion exchange is a fast physiological-chemical process that is affected e.g., by the number and type of free cation exchange sites, the age of the cells and their reaction to desiccation, growing conditions, temperature, precipitation pH, composition of the pollutants and leaching (Tyler 1990, Brown & Brûmelis 1996). In the ion exchange process, cations and anions become attached to functional organic groups in the cell walls among other things through chelation (Rao 1984).

The physiological processes affecting accumulation in lichens have been studied much more than for many other biomonitors. Attempts have also been made to explain the accumulation processes with the aid of mathematical models (Reis et al. 1999). These studies have emphasised the significance of lichen morphology and physiology in the accumulation of elements (Brown 1991, Sloof & Wolterbeek 1991). Clear differences in the accumulation of elements have been found between different lichen species and even different populations as a result of these morphological and physiological differences (Sloof 1995, Bennett & Wetmore 1999). Sporadic desiccation of lichens may

have an effect on the accumulation and absorption of elements (Puckett 1988). After a dry period, rainfall may result in appreciable washing off of particles and the exchange of cations bound on negatively charged exchange sites on the cell walls and plasma membranes of the cells (Bargagli 1998).

There are a considerable number of factors, associated with the site where lichens are growing, which may change the concentrations of pollutants in lichens (Brown 1991, Garty 2000). These factors are: quality of the deposition (form of occurrence, composition, pH), climate (composition of precipitation, temperature, wind, drought, length of the growing period) and local environmental factors (vegetation, quality of the substrate, stand throughfall and stemflow, dust derived from soil, altitude of area). On the other hand, throughfall and stemflow, which vary according to the type of canopy cover, have a greatest effect on epiphytic lichens (Barkman 1958, Rasmussen 1978). Nutrients and other elements may pass from the substrate into lichens (De Bruin & Hackenitz 1986, Bargagli 1990, Wolterbeek & Bode 1995).

Environmental biomonitoring of POPs have been performed over the years, either in terrestrial or aquatic environments, using living organisms, such as vegetation (pine needles, leaves, grass, vegetables, etc.), birds, fish, molluscs (Lovett et al., 1997; Buckley-Golder, 1999; Coutinho et al., 1999; Sakurai et al., 2000; Senthilkumar et al., 2002; Srogi, 2007: Domingo et al., 2000, 2001a,b; Schuhmacher et al., 2002). Though lichens have been the most used biomonitors in terrestrial environments to assess atmospheric deposition of a wide range of pollutants, only a few studies have used these organisms to assess POP atmospheric deposition (Augusto et al., 2004; Guidotti et al., 2003; Domeño et al., 2006; Blasco et al., 2006). Aquatic bryophytes, sharing most of the monitoring features with lichens, have also been used to monitor a large set of pollutants in aquatic environments, but specifically regarding POPs, only a limited number of studies have been performed (Roy et al., 1994, 1996; Sérgio et al., 1992; Vieira et al., 2009).

1.9. Aims & Outline

Though many studies have been performed using these organisms as biomonitors of a wide range of pollutants (such as heavy metals, radionuclides, gaseous pollutants, etc.), their use as POP biomonitors is still in a germinal stage and needs further study.

The main aim of this thesis is study the factors that influence the interception and accumulation of POPs by lichens, how lichens and aquatic bryophytes can be used to track different pollution sources in terrestrial and aquatic environments, and how can these biomonitors contribute to environmental health studies. This work will integrate different types of knowledge; allow developing an integrated biomonitor technology to be used to assess environmental POP pollution.

For that, after this **first chapter** of general introduction, the thesis was divided into 4 chapters (from second to fifth).

In the **second chapter** (*Optimizing and inter-calibrating biomonitors, soil and air*) the aim is to optimise the use of lichens as POP biomonitors. Lichens have been used during decades as metal and other pollutant biomonitors, but to date only a few studies were performed using lichens to monitor POP environmental pollution. In this way, it was necessary to study the factors that contribute for the interception and accumulation of PCDD/Fs and PAHs in lichens. Questions regarding the influence of lichen traits (growth form, size, age) and methodological aspects (influence of substrate) on their performance as POP biomonitors will be answered (subchapter **2.1**). In this second chapter, lichens are also compared with other monitoring methods, such as the use of pine needles, air and soil samples (subchapters **2.2** and **2.3**). Calibrations between POPs in lichens and air and soil are also performed (subchapters **2.3** and **2.4**), allowing translating values in lichens into the equivalent ones for air and soil. This calibration allows the integration of lichens in regulatory monitoring schemes (subchapter **2.4**).

One of the major challenges in environmental monitoring studies is to track pollution sources. This can be a tricky task in multisource environments, where different kinds of industries, urban activities, agricultural practices, etc., are all contributing to the input of POPs in the environment. The aim of the **third chapter** (*Fingerprinting pollution sources using biomonitoring tools*) is to show how lichens and aquatic bryophytes can be used to identify different pollutant sources in terrestrial (subchapter **3.1**) and aquatic environments (subchapter **3.2**). For that, an integrated analysis of different levels of information will be presented. Information regarding POP and heavy metal concentrations in biomonitors, ratios between different POP compounds, and land-use will be used together in the same approach in order to identify the origin of POP pollution.

In the **fourth chapter** (*Assessing environmental and human health risk based on integration of different monitoring approaches*) it is shown how biomonitoring using lichens can be used to complement human health studies (subchapters **4.1** and **4.2**). In environmental health studies, when the aim is to relate pollution to human health, one of the major limitations is to assess which populations should be considered as control and which ones should be considered exposed. This limitation is a consequence of the lack of spatial resolution of pollutant deposition data. Usually, data from a single air quality monitoring station is considered as representative of a large area, and thus the level of human exposure to pollutants is considered to be the same all over the region. The use of environmental biomonitors can be helpful, as they allow obtaining pollution data with high spatial resolution. In the particular case of POPs, using lichens as long-term accumulators will provide information regarding the human chronic exposure to POPs through inhalation.

Finally, the **fifth chapter** (*General discussion*) consists of a general discussion, where all the know-how acquired during the previous chapters will be integrated and interpreted. In this chapter it will be also proposed a set of guidelines to be followed when using lichens and aquatic bryophytes as biomonitors of persistent organic pollutants.

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Chapter 02 |

Optimizing and inter-calibrating biomonitors, soil and air

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Lichens as an integrating tool for monitoring PAH atmospheric deposition: a comparison with soil, air and vegetation. Augusto S, Máguas C, Matos J, Pereira MJ, Branquinho C. 2010. Environmental Pollution 158(2): 483-489.

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2.1 | Understanding the performance of different lichen species as biomonitors of atmospheric dioxins and furans: potential for intercalibration

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ABSTRACT

The aim of this study was to compare the performance of two lichen species—*Xanthoria parietina* and *Ramalina canariensis*—as biomonitors of the toxic organic compounds dioxins and furans (PCDD/Fs). For that purpose, the concentrations and profiles of PCDD/Fs found in samples of these two lichen species were compared. Results showed that *R. canariensis* presented higher concentrations than *X. parietina* and that the PCDD/F homologue profiles were substantially different between these species. *Xanthoria parietina* appeared to be a more efficient interceptor of more chlorinated PCDD/Fs and also of particles, whereas *R. canariensis* mainly reflected less chlorinated PCDD/Fs. Results also showed that the PCDD/F profile of *X. parietina* differed from the one found in other foliose and fruticose lichen species. Despite the differences observed between the profiles of *R. canariensis* and *X. parietina*, the calibration of PCDD/F concentrations between the two species was achieved, allowing the biomonitoring of PCDD/Fs.

INTRODUCTION

Polychlorinated dibenzo-p-dioxins and polychlorinated dibenzo-p-furans (PCDD/Fs) are a group of more than 200 organic compounds, which are unwanted by-products of combustion in many industrial chemical processes. These compounds are very important in environmental health studies because they are carcinogenic and potentially toxic, even at very low concentrations (WHO 2007).

PCDD/Fs can be divided according to their homologue groups (from TCDD/Fs, with four chlorine atoms to OCDD/Fs, with eight chlorine atoms), and to their congener groups (which vary with the position of chlorine atoms in the molecule); there are 17 toxic congeners with at least 4 chlorine atoms in the positions 2, 3, 7 and 8 of the molecule. The relative proportion of each group can be useful to identify different pollution sources because these have specific PCDD/F signatures in terms of the proportion of homologue and congener profiles (Cleverly et al. 1997).

Because of their chemistry, PCDD/Fs are lipophilic and very persistent in the environment, and have been detected in almost all environmental matrices, such as soil, sediment, air, water, vegetation and animals (WHO 1992; Fiedler 1990). The European

Union recommended the development of indicators to monitor the impact of regulatory controls on future levels of human exposure to PCDD/Fs, particularly concentrations in air and deposition (Buckley-Golder 1999). Because most PCDD/Fs occur at very low concentrations that vary considerably in space and time, they are difficult to assess. Using biomonitors for these measurements presents advantages, since certain types of biological organisms accumulate the pollutants to be assessed, providing a measure which integrates the exposure over a given time. Numerous types of organisms have been used to monitor PCDD/Fs, including vegetation (pine needles, leaves, grass, vegetables, etc.), birds, fishes, mollusks and more recently lichens (Lovett et al. 1997; Buckley-Golder 1999; Coutinho et al. 1999; Sakurai et al. 2000; Senthilkumar et al. 2002; Augusto et al. 2004a, b, 2007a, b).

Lichens are long-lived, extremely sensitive symbiotic organisms consisting of fungi and algae, and are the most studied biomonitors of air pollution. Recent work on their performance as biomonitors of organic compounds has shown the potential of these organisms to monitor PCDD/F atmospheric deposition (Augusto et al. 2004a, b, 2007a, b). Two different lichen species have been used in these studies: *Xanthoria parietina* (L.) Th. Fr., a flat, leaf-like lichen, with well-defined upper and lower surfaces, broadly attached to the substrate (foliose lichen); and *Ramalina canariensis* Steiner, a densely branched and ‘‘three dimensional’’ lichen form, with a single-point of attachment (fruticose lichen). Both species are ubiquitous but they generally occupy sites with different land uses. Whereas *X. parietina* is a very tolerant species that can be found in highly polluted sites, such as in urban and industrial areas, *R. canariensis* is found in forests and natural areas. In biomonitoring studies requiring a high density of sampling sites and a regional-scale cover, where a high diversity of land uses may occur, it is difficult to find the same lichen species over the whole territory. In such cases, the intercalibration between two or more lichen species is important to avoid gaps in the sampling grid.

Although *X. parietina* and *R. canariensis* have been successfully used as PCDD/Fs biomonitors, their intercalibration still requires some further study. Thus, the aim of the present work is to understand the different patterns of accumulation of PCDD/Fs in these lichens (namely the effects of the growth form, substrate and type of particle interception) and to intercalibrate the two species.

EXPERIMENTAL SECTION

Sampling

For calibration purposes, samples of the lichens *R. canariensis* and *X. parietina* (8–12 g, each sample) were collected at eight sites in Setúbal peninsula, a region selected in Portugal, which is an important urban and industrial area of the country. At each of the sampling sites, *R. canariensis* was collected from five to ten stone pines (*Pinus pinea* Aiton), always at a 1–3 m height, and *X. parietina* was collected from the available house roof tiles (3 m height). In order to compare the concentrations and profiles of PCDD/Fs found in *R. canariensis* and *X. parietina* with the average profile of other lichen species, in one of the sampling sites the fruticose lichen *R. fastigiata* and the foliose lichens *Parmelia caperata* (L.) Hale and *Parmotrema reticulatum* (Taylor) M.Choisy were also sampled. All of these samples were collected from olive and stone pine.

In order to test the influence of the substrate (roof tiles or type of phorophyte) on the levels and profiles of PCDD/Fs in lichens, a sample of *X. parietina* was collected from house roof-tiles and from olive branches (*Olea europaea* L.) at a ninth sampling site; at a tenth sampling site, samples of *R. canariensis* were collected from olive, cork oak (*Quercus suber* L.) and stone pine, and a sample of *Ramalina fastigiata* (Pers.) Ach. was collected from olive, cork-oak and Portuguese oak (*Quercus faginea* Lamk).

Sample analysis

After collection, all samples were stored in plastic bags and transported to the laboratory, where unwashed samples were immediately dried at room temperature and sorted to remove extraneous material. The cleaned samples (c. 15 g) were then ground, kept in closed glass containers and analyzed for PCDD/Fs. The glass containers were kept at room temperature, between 20 and 25C. The PCDD/F analysis was performed following the EPA 1613 B protocol and took place in the specialized analytic laboratory TERRA PROTECTA in Berlin, Germany, which has a German Accreditation for Dioxin Measurements. For metal analysis, ground lichen samples of approximately 100 mg dry weight (dried at 50ºC for 24 h in a hot air oven) were digested with 3 ml of nitric acid (65%) at 120ºC. Glass tubes with 3 ml of nitric acid and without lichen samples were used as controls. Zinc, Fe, Mg, Mn, Ca and K were analyzed by atomic absorption spectrophotometry (SpectrAA/50 Varian), using an air/acetylene mixture flame. Before Ca and K analysis, CsCl and LaCl3 (1 g/l) were added to the samples to prevent

ionization and the formation of refractory compounds. Lead, Cr, Co and Cu were analyzed by atomic absorption spectrophotometry (GBC 932 plus) using a graphite furnace (GBC GF 3000).

The analytical accuracy of the results was checked against the reference material IAEA-336 (Stone et al. 1995). The results of the analyzed elements were within the confidence intervals of the certified values. Detection limits were 1.0 μg/l for Zn, 6.0 μg/l for Fe, 0.3 μg/l for Mg, 2.0 μg/l for Mn, 1.0 μg/l for Ca, 3.0 μg/l for K, 0.3 μg/l for Cu, 0.28 μg/l for Pb, 0.21 μg/l for Co and 0.075 μg/l for Cr.

Statistical analysis

Summary statistics (median, standard deviation, minimum and maximum) were used to characterize PCDD/F and metal concentrations determined in all the collected samples. Pearson linear correlations between chemical element concentrations and PCDD/F homologues for the target lichen species (*R. canariensis* and *X. parietina*) were calculated. A 5% level of significance (P = 0.05) was considered for the results.

RESULTS AND DISCUSSION

The concentrations of PCDD/Fs in lichens collected at the same sites ranged between 170.8 and 344.7 ng/Kg in *X. parietina* and from391.9 to 1058.6 ng/Kg in *R. canariensis* (Table 1). The fruticose lichen *R. canariensis* seemed to accumulate higher concentrations of total PCDD/Fs when compared to the foliose lichen *X. parietina*. On the other hand, *X. parietina* showed higher concentrations for all the metals analyzed except for Ca (Table 2). Lichens produce a wide range of secondary products, many of which occur as extra cellular crystals within the thallus; the highest concentration of Ca in the lichen *R. canariensis* can be of biogenic origin, rather than a result of environmental pollution (Gaio-Oliveira et al. 1999; Oliveira et al. 1998).

Lichen morphology influences the rate at which lichens accumulate elements from the atmosphere (Garty 2001). Growth form dictates thallus orientation and the amount of continuous surface area exposed to airborne deposition; therefore, it should have a direct impact on the interception of atmospheric elements by lichens. *Ramalina canariensis* has a bushy-like structure, with a higher surface/volume ratio than *X. parietina*. This characteristic might facilitate the interception of aerosols and low

molecular weight particles by *R. canariensis*. Another explanation for the higher values of PCDD/Fs in *R. canariensis* may be related to specific characteristics of this lichen's surface that contribute to the retention of lipophilic compounds. For lichens, no information on the ability to accumulate organic compounds is available but there are several reports concerning their high efficiency to intercept and retain atmospheric particles, especially the smallest ones (Branquinho 1997, 2001; Branquinho et al. 1999). More than 50% of the total chemical content in lichens might be in the particulate form, trapped within the fungal hyphae and retained there for very long periods. Features such as roughness and gelatinous surfaces may facilitate the interception, uptake and retention of PCDD/Fs bound particles or of PCDD/Fs in the gas-phase.

TABLE 1. Statistical summary of PCDD/F concentrations (ng/Kg) in lichen samples collected at the same sites and at the same time.

	Mean	SD	Minimum	Maximum
X. parietina	246.3	65.5	170.8	344.7
R. canariensis	799.7	231.5	391.9	1058.6

$N = 8$ sites

TABLE 2. Statistical summary of metal concentrations (mg/Kg) in lichens collected at the same sites and at the same time.

mg/Kg	*X. parietina*		*R. canariensis*	
	Mean	SD	Mean	SD
Zn	69.5	21.5	42.1	19.3
Fe	2051.4	964.3	518.8	221.1
Mg	1095.6	324.0	574.1	175.0
Mn	54.3	48.5	21.6	4.9
Ca	1364.8	614.9	5109.9	4089.1
K	3200.4	1938.3	1775.0	1104.5
Cu	23.4	15.7	13.6	4.7
Pb	4.0	2.5	3.0	2.5
Co	13.3	12.4	11.5	10.9
Cr	67.9	87.7	8.5	3.9

$N = 8$ sites

Comparing the homologue profiles of the two species, it can be observed that the profile in *X. parietina* is dominated by the more chlorinated PCDD/Fs, such as OCDD, OCDF, HpCDD and HpCDF, whereas in *R. canariensis* the profile is dominated by the less chlorinated PCDD/Fs (Figure 1a).

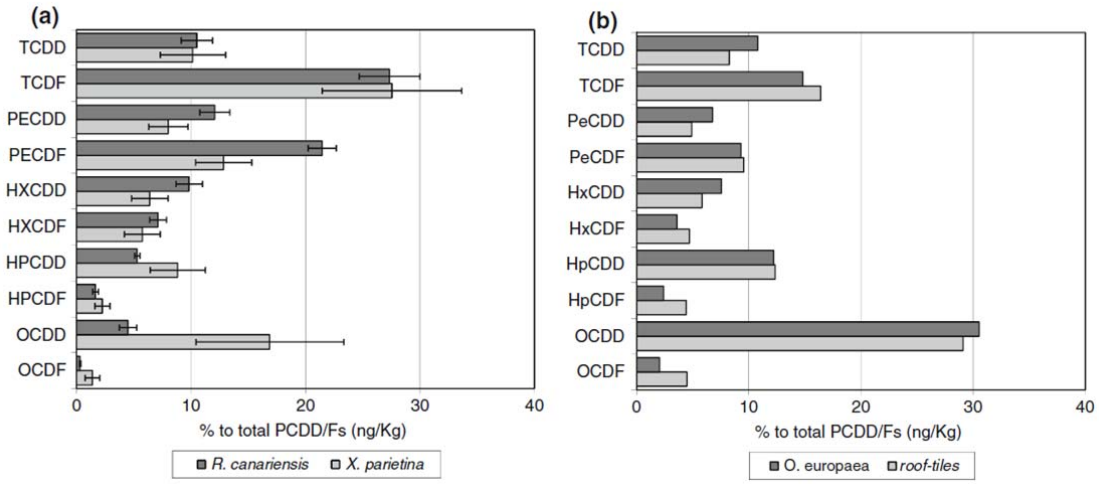

Figure 1. a) PCDD/F homologue profiles in the lichens *Ramalina canariensis* and *Xanthoria parietina*. Percentage contribution of each homologue to the total PCDD/Fs. N=8 sites. Error bars represent SD. b) PCDD/Fs homologue profile in *X. parietina* collected from roof-tiles and from olive in the same sampling site. Percentage contribution of each homologue to the total PCDD/Fs. N=1 site.

These differences are not likely to be due to differences in the substrate from where the samples were collected— roof-tiles and pine—since *X. parietina* samples collected from roof-tiles and from olive trunks showed no differences between homologue profiles (Figure 1b). Moreover, samples collected from roof-tiles showed higher concentrations of PCDD/Fs than those collected from olive (755.9 and 442.1 ng/Kg, respectively). Regarding the fruticose lichens, to test the influence of different substrates on the concentrations and profile of PCDD/Fs, we collected lichens of the species *R. canariensis* and *R. fastigiata* from different phorophytes (tree species). The variation of PCDD/F concentrations was 7.6% for *R. canariensis* and 3.0% for *R. fastigiata*, showing that they were not affected by the type of phorophyte from where the samples were collected. The PCDD/F homologue profiles are displayed in Figures 2a, b, and show that PCDD/F profiles were very similar among samples collected from different phorophytes.

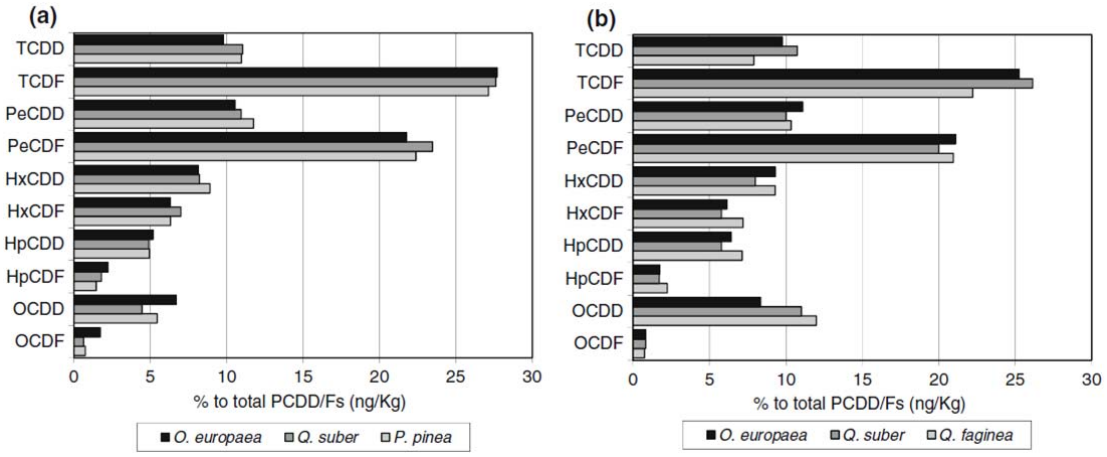

Figure 2. a) PCDD/Fs homologue profile in the lichen *R. canariensis* collected from three different phorophytes: *P. pinea*, *Q. suber* and *O. europaea*. Percentage contribution of each homologue to the total PCDD/Fs. b) PCDD/Fs homologue profile in the lichen *R. fastigiata* collected from three different phorophytes: *Q. faginea*, *Q. suber* and *O. europaea*. Percentage contribution of each homologue to the total PCDD/Fs.

Researches on uptake by lichens suggest that lichens may take up elements from the substrate (Goyal and Seaward 1981; Prussia and Killingbeck 1991; Loppi et al. 1999). Before starting a biomonitoring survey, it is therefore necessary to test the effect of different phorophytes on the levels of pollutants found in their epiphytic lichens (Oliveira et al. 1998; Prussia and Killingbeck 1991). If differences are observed between phorophytes, care should be taken to choose only one type or to intercalibrate lichen concentrations between phorophytes. Branquinho (1997) has shown that elements in lichens are more related to atmospheric driven particles than to the through fall of the tree canopy.

In this study no differences were found among PCDD/F concentrations and profiles in lichens collected from different substrates, indicating that the substrate is apparently not a source of variability when sampling lichens for PCDD/F monitoring purposes.

Results of the correlation analysis between the metal content and percentage contribution of each homologue to the total PCDD/Fs in lichens are displayed in Table 3. In *X. parietina*, the contribution of the more chlorinated PCDD/ Fs was positively correlated with metals such as Zn, Fe, Mn, Co and Cr; and the contribution of the less

chlorinated PCDD/Fs was negatively correlated with metals. In *R. canariensis* this pattern of correlations was inexistent.

These results clearly showed that *X. parietina* and *R. canariensis* have different patterns of interception and accumulation of PCDD/Fs and metals. Whereas *X. parietina* mainly reflected the more chlorinated PCDD/Fs, *R. canariensis* mainly reflected the less chlorinated PCDD/Fs. Some authors argued that the more chlorinated PCDD/Fs are more stable in the environment than the less chlorinated PCDD/Fs (Domingo et al. 2001a, b). The strongest contribution of the more chlorinated PCDD/Fs in *X. parietina* can be related to the high longevity of this lichen species. In experiments where the levels of PCDD/Fs in younger and older parts of thalli of *X. parietina* were compared, it was found that older parts presented higher levels of OCDD (unpublished results).

TABLE 3. Correlations between metal contents and percentage contributions of each homologue to the total PCDD/Fs in lichens of the species *X. parietina* and *R. canariensis*. N = 8 sites. Marked correlations are significant at P<0.05.

		OCDF	OCDD	HPCDF	HPCDD	HXCDF	HXCDD	PECDF	PECDD	TCDF	TCDD
X. parietina	Zn	**0.75**	0.46	**0.82**	0.43	**0.79**	−0.11	−0.22	−0.36	−0.46	**−0.73**
	Fe	0.69	0.60	**0.81**	0.53	**0.83**	0.04	−0.51	−0.51	−0.54	**−0.72**
	Mg	−0.11	−0.27	−0.16	−0.25	−0.42	−0.17	0.64	0.21	0.25	0.00
	Mn	0.51	**0.79**	**0.73**	**0.73**	0.69	0.14	−0.67	−0.64	−0.67	**−0.76**
	Ca	0.25	0.40	0.38	0.48	0.41	−0.18	0.08	−0.32	−0.41	−0.58
	K	−0.25	−0.01	−0.08	0.04	0.40	−0.43	0.03	−0.30	0.14	−0.06
	Cu	−0.04	0.00	0.15	0.27	0.33	−0.47	−0.07	0.58	−0.20	−0.04
	Pb	0.59	0.15	0.59	0.31	0.68	−0.33	0.02	0.28	−0.39	−0.40
	Co	0.62	**0.86**	**0.84**	**0.87**	**0.83**	0.11	−0.67	−0.57	**−0.82**	**−0.86**
	Cr	0.29	0.61	0.57	0.66	**0.94**	−0.22	−0.67	−0.50	−0.52	−0.55
R. canariensis	Zn	**0.89**	**0.82**	0.28	0.70	**−0.74**	−0.70	**−0.80**	**−0.73**	0.61	0.13
	Fe	−0.19	−0.04	−0.33	−0.10	−0.03	−0.20	−0.10	−0.10	0.31	0.13
	Mg	−0.03	0.01	0.02	−0.14	0.05	−0.08	−0.37	−0.31	0.38	0.41
	Mn	−0.06	−0.15	−0.08	−0.10	0.03	0.25	0.03	0.39	−0.22	−0.10
	Ca	−0.09	−0.11	0.24	−0.24	0.18	0.03	−0.06	−0.23	0.33	−0.14
	K	0.43	0.46	0.11	0.32	−0.30	−0.49	−0.66	−0.35	0.27	0.70
	Cu	0.41	0.37	0.13	0.28	−0.34	−0.42	−0.36	−0.10	0.14	0.19
	Pb	−0.35	−0.37	0.02	−0.44	0.25	0.15	0.42	0.12	0.09	−0.41
	Co	−0.31	−0.02	**−0.89**	−0.06	−0.23	−0.40	−0.07	−0.05	0.27	0.67
	Cr	−0.36	−0.22	−0.37	−0.19	0.15	0.08	0.08	0.15	0.08	−0.02

$N = 8$ sites. Marked correlations are significant at $P < 0.05$

Apparently, the PCDD/F profile displayed by *X. parietina* presents similarities to those detected in soil samples, which are also dominated by the more chlorinated compounds and reflect long-term exposure and accumulation (Ogura et al. 2001a, b). Lichens are

long-lived organisms that can accumulate pollutants over decades and it should therefore be expected that both studied species presented a major contribution of the more chlorinated PCDD/Fs. However, the difference between profiles indicates that perhaps the structure of the lichen thallus—foliose and fruticose—also plays a role in the interception and accumulation of PCDD/Fs. In order to test this hypothesis, samples from other foliose and fruticose lichen species were collected and analyzed for PCDD/Fs. The results are displayed in Figure 3.

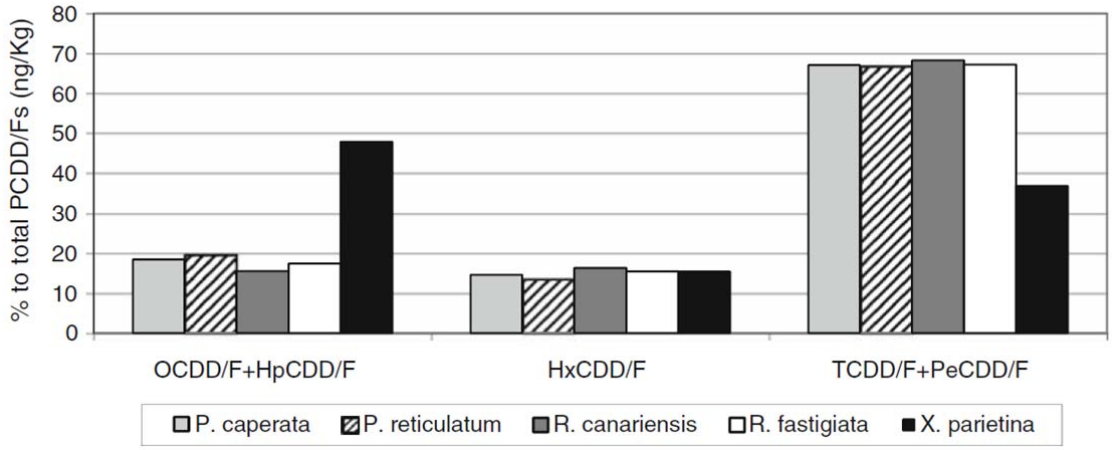

Figure 3. PCDD/F homologue profiles in the foliose lichens *P. caperata*, *P. reticulatum* and *X. parietina*, and in the fruticose lichens *R. canariensis* and *R. fastigiata*. Percentage contribution of each homologue to the total PCDD/Fs. N = 1 site. The class OCDD/F+HpCDD/F includes the homologues OCDD, OCDF, HpCDD, HpCDF and HxCDD/F; HxCDD/F includes the homologues HxCDD and HxCDF; TCDD/F+PeCDD/F includes the homologues TCDD, TCDF, PeCDD and PeCDF.

The PCDD/F profiles detected in all samples of foliose (*P. caperata* and *P. reticulatum*) and fruticose lichens (*R. fastigiata*) were similar to the ones found for *R. canariensis*, with the less chlorinated compounds dominating the profile. Apparently, *X. parietina* showed a unique PCDD/F profile (with the more chlorinated compounds dominating) when compared to other species, a fact which does not seem to be related with the structure of the lichen thallus.

Element uptake by thalli depends on several ecological factors, such as the nature of the element, morphological features and environmental parameters (Garty 2001). Trace

elements are deposited onto the lichen surface either as dry particulate or as material dissolved and/or suspended in precipitation.

These elements may be retained by particulate entrapment, physicochemical processes such as ion exchange, and by passive and active intracellular uptake (Tyler 1989). In the case of *X. parietina*, the high concentrations of metals associated to the more chlorinated PCDD/Fs may indicate that a certain proportion of the lichen elemental composition may derive from soil particulates (Guevara et al. 1995). Metals, such as Al and Fe, once deposited on the lichen surface, accumulate by particulate entrapment (Loppi et al. 1999) since oxides of these elements are relatively insoluble in atmospheric particulates (Mastino et al. 1987) and do not substantially accumulate by processes requiring water solubility.

Attempting to intercalibrate *R. canariensis* and *X. parietina*, bi-plots were drawn for the total concentrations of PCDD, PCDF and PCDD/Fs (Figure 4).

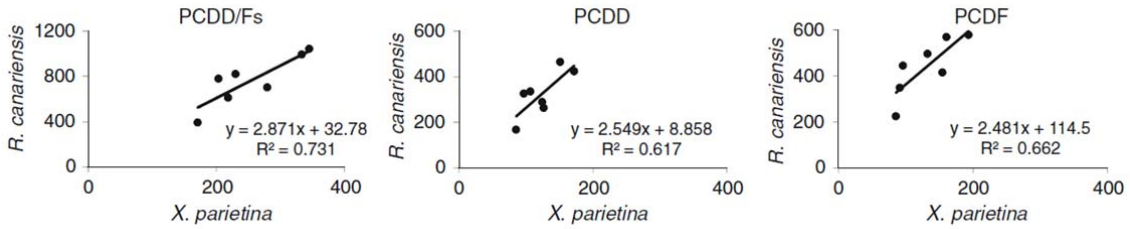

Figure 4. Correlation of PCDD/Fs, PCDD and PCDF in the lichens *X. parietina* and *R. canariensis*, where R is the correlation coefficient. N = 7 sites (one outlier excluded).

Although these species display different patterns of PCDD/Fs accumulation, a significant linear correlation between the total concentrations of PCDD, PCDF and PCDD/Fs can be found in both. This could be useful when biomonitoring studies regarding PCDD/Fs are intended, but the same lichen species cannot be found over the entire study region. This is very common in regions with a variety of land-uses, including industrial, urban and forestry areas. Although it could be easy to find lichens growing in forest areas, in urban and industrial areas lichen diversity is usually very low. *Xanthoria parietina* is a very tolerant lichen species which can be found growing in such areas. In this sense, *X. parietina* and *R. canariensis* could be used for monitoring PCDD/F concentrations. Using the lichen X*. parietina* as a biomonitor, Augusto et al. (2004b) found that PCDD/F

deposition occurred mainly in industrial and highly populated urban areas. The same authors found that the PCDD/F profiles detected in *R. canariensis* reflect air pollution rather than soil contamination (Augusto et al. 2007b).

CONCLUSIONS

The comparison between homologue profiles of *X. parietina* and *R. canariensis* revealed substantial differences. *Xanthoria parietina* showed to be a more efficient interceptor of the more chlorinated PCDD/Fs, which may be related to a greater interception of particles from the soil, whereas *R. canariensis* mainly reflects the less chlorinated PCDD/Fs. Nevertheless, a significant calibration between the two species was achieved for PCDD, PCDF and PCDD/F concentrations, allowing the biomonitoring of these compounds at a regional scale (comprising a wide variety of land uses, and a dense sampling grid) using both species.

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2.2 | Interpreting the dioxin and furan profiles in the lichen *Ramalina canariensis* Steiner for monitoring air pollution

Published in Science of the Total Environment (2007) 377:114-123

ABSTRACT

The purpose of this study was to compare the dioxin and furan (PCDD/F) profiles in lichens with those of air and soil. Lichen samples of the species *Ramalina canariensis* Steiner were collected from 44 different sites and analysed. The results were compared to PCDD/F air and soil profiles from bibliographic data concerning several countries and locations. When compared to other biomonitors (pine needles, vegetation, fruits), lichens were observed to accumulate greater concentrations of PCDD/Fs. The results showed that, although the concentrations of PCDD/Fs in lichens were at the same level of magnitude as those found for soils from the same country (197.5–1218.7 ng kg^{-1} and 2.3–15.2 ng I-TEQ kg^{-1}), the PCDD/Fs profiles do not reflect soil particle contamination. On the contrary, the PCDD/Fs lichen profiles seemed to be very similar to the ones in the air, at least for the diversity of the ones used for comparison in this study. These results indicated that lichens of the species *R. canariensis* are potential biomonitors of PCDD/F air pollution.

INTRODUCTION

Worldwide contaminations by dioxins and related compounds, such as polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) have caused great concern due to their persistency in the environment. Because most PCDD/Fs occur at very low concentrations that vary considerably in space and time, they are difficult to measure. The use of biomonitors to perform these measurements has advantages, since certain types of biological organisms become enriched in the pollutants to be assessed, providing a measure of integrated exposure over a given time. Numerous biomonitors have been used to monitor PCDD/Fs, including vegetation (pine needles, leaves, grass, vegetables, etc.), birds, fishes, and molluscs (Lovett et al., 1997; Buckley-Golder, 1999; Coutinho et al., 1999; Sakurai et al., 2000; Senthilkumar et al., 2002). To date, pine needles are the most used biomonitors to evaluate air deposition of PCDD/Fs, as they can be found worldwide, allowing comparisons between countries. While vegetation is mainly used to provide information on the short-term exposure to PCDD/Fs, soil samples are also commonly analysed in order to describe long-term exposure to PCDD/Fs, since soil is a sink for these compounds (Domingo et al., 2000, 2001a; Schuhmacher et al., 2002).

Lichens, symbioses of fungi and algae or cyanobacteria, have been extensively used to biomonitor a variety of pollutants, given their ability to accumulate both particulate material in extra- and intracellular sites and soluble chemicals, most of them by cation-exchange capacity in the cell wall. These pollutants have been related to air pollutant concentrations (Garty, 1993; Branquinho, 2001). This relation is mainly a result of the fact that they have no roots, and consequently they are very efficient in taking up particles (which also have nutrients) and by consequence pollutants, where they occur, directly from the atmosphere. They are also very efficient in absorbing water vapor, since they are poikilohydric organisms. Additionally, their ubiquity, long life, slow growth and constant morphology throughout the year contribute to make them excellent biomonitors (for reviews see Brown and Avalos, 1991; Garty, 1993; Richardson, 1992; Bargagli, 1998). The following are among the pollutants measured in lichens: sulfur, nitrogen, fluoride, metals, radionuclides, and a variety of organic compounds like PCBs (polychlorinated biphenyls), PAHs (Polycyclic Aromatic Hydrocarbons), and substances from organochloride pesticides (HCHs and HCB) (Villeneuve et al., 1988; Herzig, 1989; Calamari et al., 1991; Garty, 2000; Owczarek et al., 2001; Guidotti et al., 2003). Lichens have also been recently used to monitor PCDD/Fs (Augusto et al., 2004), however more information is needed to interpret the concentrations and profiles of PCDD/Fs in lichens. Lichens as biomonitors have been shown to reflect mainly atmospheric deposition; whereas some authors have reported contamination from soil mainly by soil particles resuspension (Bargagli, 1990). In this way, evaluating what are lichens PCDD/Fs reflecting (air or soil) is of obvious importance for using these biomonitors as reliable tools in environmental monitoring and environmental chemistry. Due to the poikilohydric nature of lichens and to the fact that they are good biomonitors of atmospheric pollution for several pollutants, it was hypothesized that the PCDD/Fs profiles in the lichen *Ramalina canariensis* Steiner reflected mainly the PCDD/F profiles of the air.

EXPERIMENTAL SECTION

Study area

The area selected to perform this study was the Setúbal peninsula, located in southern Portugal, covering an area of 150,000 ha, with the Atlantic Ocean as its western limit

(Figure 1). This region is one of the most industrialized and densely populated in the country. Among the industries present in the region, iron and steel plants, cement mills, power plants, several chemical plants and hospital incinerators can be highlighted. This region is simultaneously rich in natural areas, notably Mediterranean woodlands, salt-marshes and rocky and sand beaches, some of them with preserved sand dunes.

Lichen species

The lichen species selected to perform this study was *R. canariensis* Steiner. This is an epiphytic (growing in tree twigs and bark), fruticose and bushy-like structured lichen. This species was selected because of several features: it is easily found in the study area and its collection is quick and simple. Its position on the branches and trunks (attached by a single point of fixation) allows the whole lichen to be exposed to air pollutants.

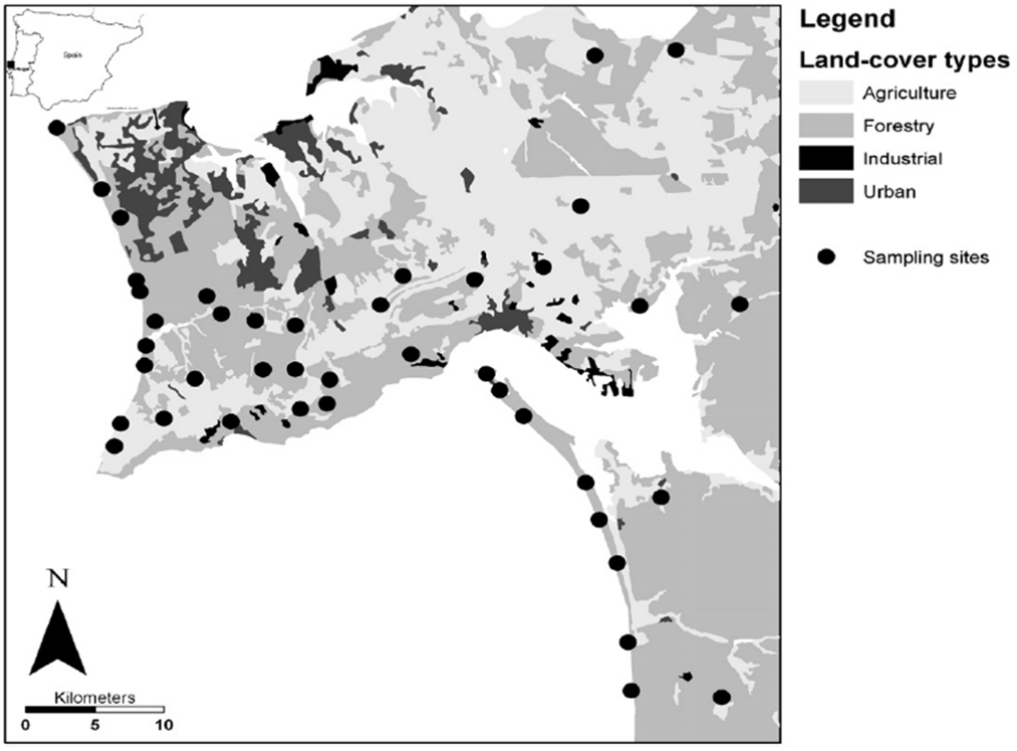

Figure 1. Land cover map of the study area, Setúbal peninsula (developed by the Life Environment Program ENV/P/000556 and Life Nature Program 98-NAT/P/5235), with the distribution of the 44 sampling sites where the lichen *Ramalina canariensis* was collected (represented by dark points).

Sampling

To achieve our goals, we sampled lichens at 44 sites in the selected region (Figure 1). Lichens were collected mainly from *Pinus pinea* Aiton, on a minimum of five to ten trees at each sampling point, and always at a 1–3 m height. At each sampling site, collection of lichens was restricted to a square area no larger than 50×50 m and no smaller than 10×10 m. Whenever possible, collections were performed at least 300 m far from main roads and at least 100 m from any secondary road. Collections were made without gloves and lichens were kept in plastic bags during transport to the laboratory. Lichen sampling was performed during five uninterrupted days of March 2000, after a dry period of 84 days (precipitation below 7 mm) and during a meteorologically stable period. In order to evaluate the within-site variability, two samples were collected at the same sampling site and each one treated independently.

PCDD/F analysis

In the laboratory, unwashed samples of lichens (whole lichens) were immediately dried at room temperature and sorted to remove extraneous material. Clean samples (c. 15 g) were then ground (Glen Creston Ltd. MM 2000), kept in closed glass containers and analysed for PCDD/Fs. The glass containers were kept at room temperature, between 20 and 25 °C. The PCDD/Fs analysis was executed following the EPA 1613 B protocol. Ground and dried lichen samples were extracted using Soxhlet method (toluene). Cleanup was based on mixed silica column, aluminum oxide column and gel-chromatography (Bio-Beads S-X3A) method. Measurement of dioxins was performed using gaseous chromatography and high-resolution mass spectrometry (HRGC/HRMS): Varian 3400 gas chromatograph equipped with a cold injection system (Gerstel KAS) and a DB-Dioxin column; Finnigan MAT 90 HRMS at a resolution 8000–10,000. Using isotope dilution method, 13C12-labeled internal standards were added prior to the extraction; the surrogate for determination of the recovery ratio was added just prior to analysis. Recovery ratios of the labeled internal standard added to each sample prior to the extraction complied well with the tolerable range of 60–120% for all samples analysed. The method used is accredited according to ISO 17025 standard (EN 45001, 2002) covering all required QA/QC measures such as, blank controls, certified reference materials, inter-laboratory comparisons etc. The analysis took place in the specialized analytic laboratory TERRA PROTECTA in Berlin, Germany, which has a German Accreditation for Dioxin Measurements. This laboratory has experience with mosses and

vegetables, being also a partner lab in a European monitoring project for mosses (*Brachythecium rutabulum*) for analysing PCDD/PCDFs.

For each sample the results obtained were:

i) the concentrations of PCDD/F homologues and toxic congeners, presented in real mass concentration (ng kg^{-1} dry weight); PCDD/Fs can be divided by their homologue groups, from TCDD/F (PCDD/F with 4 chlorine atoms) to OCDD/F (PCDD/F with 8 chlorine atoms), or by their congener groups (according to the position at which chlorine atoms are in the molecule); there are 17 toxic congeners which correspond to the congeners that have at least 4 chlorine atoms in the positions 2,3,7,8.

ii) the concentrations of the seventeen toxic PCDD/Fs congeners converted to the 2,3,7,8-TeCDD International Toxic Equivalents (I-TEQ), calculated using theNATO/CCMS factors, in order to evaluate the samples toxicity (NATO/CCMS, 1988). The TEQ is determined using a method that multiplies the value of each congener's concentration by its TEF and then sums the multiplication products for all 17 congeners. The TEF for each congener is based on the congener's toxic potency relative to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which is the most toxic congener. Thus, the congeners with the highest products of concentration and TEF would contribute the most to the overall TEQ estimate.

iii) total PCDD/Fs concentrations obtained through the sum of the concentrations of the homologues, and the total I-TEQ obtained through the sum of the I-TEQ calculated for the toxic congeners.

Air and soil data

All data used in this study regarding the air and soil PCDD/Fs profiles were obtained from a bibliography (published between 2002 and 2006). List of references is displayed in Table 1.

Data analysis

Summary statistics (average, median, and standard deviation) were used to characterize each congener and homologue of PCDD/Fs determined in the totality of the collected samples. In order to test whether the lichens were reflecting air or soil pollution, a principal component analysis (PCA) was performed using the data obtained in this study for PCDD/Fs in lichens (n=44) and data obtained from the bibliography for PCDD/Fs in

air (n=111) and soil (n=40) from different sites all over the world (Table 1). The PCA was performed using the contribution of each congener to the total PCDD/Fs.

TABLE 1. Bibliography of PCDD/Fs in air and soil.

Country	Matrix	Sites characterization	Reference
China	Air	Industrial, residential, commercial, suburban	Yu et al. (2006)
Greece	Air	Urban	Schuhmacher et al. (2002)
Taiwan	Air	Industrial	Shih et al. (2006); Turrio-Baldassarri et al. (2005); Im et al. (2004)
USA	Air	Industrial, residential, commercial, suburban, rural	Correa et al. (2006); Raun et al. (2005)
Italy	Soil and air	Industrial, urban, suburban	Park and Kim (2002); Kim et al. (2005)
Korea	Soil and air	Industrial, urban, rural, residential, commercial	Caserini et al. (2004); Mandalakis et al. (2002); Chen et al. (2004)
Spain	Soil and air	Industrial, residential	Schuhmacher et al. (2002); Schuhmacher et al. (2006)
Turkey	Soil and air	Industrial, rural, natural	Lee et al. (2005)

RESULTS AND DISCUSSION

PCDD/Fs concentrations in lichens: comparison with other matrices

The statistical summary for PCDD/Fs concentrations in the lichen *R. canariensis* is displayed in Table 2. The total PCDD/F concentrations found for this lichen ranged between 197.5 and 1218.7 ng kg^{-1} dw, with an average value of 705.1 ng kg^{-1} dw and a median of 694.1 ng kg^{-1} dw. In terms of I-TEQ, the values ranged between 2.3 and 15.2 ng I-TEQ kg^{-1} dw, with an average of 8.8 ng I-TEQ kg^{-1} dw and a median of 8.3 ng I-TEQ kg^{-1} dw (Table 2). Regarding the within site variability, the two samples of *R. canariensis* collected at the same site showed similar values for the ΣPCDD/Fs and the toxic ΣPCDD/Fs (I-TEQ), with an average of 341.2 ng kg^{-1} dw and 4.9 ng I-TEQ kg^{-1} dw respectively (Table 2).

When compared to other matrices, measured in EU member states at locations with known contamination levels, the measured range (expressed in I-TEQ) in the lichens used in this study was much greater than the typical range found for spruce/pine needles, where the average values ranged between 0.3 and 1.9 ng I-TEQ kg^{-1} dw (Buckley-Golder, 1999).When compared to the typical levels for soils from EU member states, 1–100 ng I-TEQ kg^{-1} (Buckley-Golder, 1999), lichens have shown lower values, but in the same range of concentrations found for Portuguese soils, where the levels vary between 0.79 and 16.39 ng I-TEQkg^{-1} dw (Coutinho et al., 1999).

Soils usually have high levels of PCDD/Fs, as they are natural sinks for persistent and lipophilic compounds such as PCDD/Fs, which adsorb to the soil organic carbon and,

once adsorbed, remain relatively immobile (Fiedler, 1999). Soil is considered a typical accumulating matrix with a long-term memory.

When compared to vegetables collected in the region of Oporto, in Portugal (Coutinho et al., 1999), and to fruits and vegetables collected at urban sites close to a solid waste incinerator (Lovett et al., 1997), with values ranging from 0.24 to 1.28 ng I-TEQ kg^{-1} dw and from 0.1 to 0.9 ng I-TEQ kg^{-1} dw respectively, lichens have shown greater concentrations (Table 2).

TABLE 2. Summary statistics of the pollutant data (ng Kg^{-1} dw) obtained through chemical analysis of the 44 samples of the lichen *Ramalina canariensis* collected in March 2000 in the Setúbal peninsula. In addition, a comparison between two samples collected at the same site and at the same time is tabulated.

		Totality of samples (n=44)						Comparison between samples		
		Average	Median	Standard deviation	Minimum	Maximum	Variation (%)	Sample 1	Sample 2	Average
ng kg^{-1} (dw)	**TCDD**	72.0	70.9	30.2	19.100	144.000	41.9	39.900	44.000	42.0
	PECDD	82.3	82.6	33.2	19.600	161.000	40.3	38.900	40.800	39.9
	HXCDD	65.6	68.5	23.4	16.700	110.000	35.7	26.600	32.200	29.4
	HPCDD	34.5	35.2	11.5	11.600	54.800	33.2	14.700	16.000	15.4
	OCDD	31.3	30.2	14.0	9.600	78.800	44.8	9.500	11.400	10.5
	TCDF	194.8	197.0	70.0	62.700	321.000	36.0	104.000	110.000	107.0
	PECDF	158.7	151.0	61.0	38.200	289.000	38.4	68.700	73.900	71.3
	HXCDF	52.4	51.1	21.0	13.200	96.800	40.1	19.400	23.300	21.4
	HPCDF	11.2	10.7	4.7	3.400	22.900	42.3	4.200	4.900	4.6
	OCDF	2.3	2.3	0.8	1.000	4.300	33.9	0.000	0.000	0.0
	ΣPCDD/Fs	705.1	694.1	251.8	197.500	1218.700	35.7	325.900	356.500	341.2
	2,3,7,8-TCDD	0.4	0.4	0.2	0.100	1.100	47.0	0.400	0.400	0.4
	1,2,3,7,8-PeCDD	1.1	1.2	0.4	0.250	2.150	34.4	0.700	0.750	0.7
	1,2,3,4,7,8-HxCDD	0.2	0.2	0.1	0.040	0.270	36.3	0.060	0.070	0.1
	1,2,3,6,7,8-HxCDD	0.3	0.4	0.1	0.090	0.540	32.1	0.180	0.210	0.2
	1,2,3,7,8,9-HxCDD	0.2	0.2	0.1	0.060	0.360	33.7	0.090	0.100	0.1
	1,2,3,4,6,7,8-HpCDD	0.1	0.2	0.0	0.045	0.237	33.3	0.067	0.072	0.1
	OCDD	0.0	0.0	0.0	0.011	0.079	45.0	0.010	0.011	0.0
	2,3,7,8-TCDF	0.8	0.8	0.3	0.210	1.600	43.5	0.450	0.500	0.5
	1,2,3,7,8-PeCDF	0.4	0.4	0.2	0.090	0.705	41.1	0.170	0.180	0.2
	2,3,4,7,8-PeCDF	3.6	3.4	1.4	0.950	6.350	38.3	1.950	2.100	2.0
	1,2,3,4,7,8-HxCDF	0.6	0.6	0.2	0.150	1.020	40.1	0.200	0.220	0.2
	1,2,3,6,7,8-HxCDF	0.5	0.5	0.2	0.150	0.980	39.9	0.230	0.270	0.3
	1,2,3,7,8,9-HxCDF	0.0	0.0	0.0	0.000	0.030	110.6	0.010	0.010	0.0
	2,3,4,6,7,8-HxCDF	0.3	0.3	0.1	0.100	0.570	35.6	0.150	0.170	0.2
	1,2,3,4,6,7,8-HpCDF	0.1	0.1	0.0	0.024	0.189	42.5	0.034	0.039	0.0
	1,2,3,4,7,8,9-HpCDF	0.0	0.0	0.0	0.000	0.005	141.8	0.003	0.003	0.0
	OCDF	0.0	0.0	0.0	0.000	0.004	39.1	0.001	0.001	0.0
	ΣPCDD/Fs (I-TEQ)	8.8	8.3	3.2	2.341	15.185	36.7	4.705	5.106	4.9

In addition, a comparison between two samples collected at the same site and the same time is tabulated.

Such substantial differences between PCDD/Fs levels in lichens and in vegetables and fruits can be due to the fact that lichens were collected in a different region, being

therefore exposed to different sources of pollution; or for the reason that plants and lichens have different ways of uptake of these compounds. Atmospheric deposition of PCDD/Fs occurs in three different forms: dry gaseous deposition, dry particle-bound deposition and wet deposition and the latter contains primarily particle bound chemicals (Mader and Pankow, 2000). Plants have been shown to accumulate PCDD/Fs due to the hydrophobic chemicals of wax cover and to the large surface area (McCrady et al., 1990; McCrady, 1994). For lichens no information on the ability to accumulate organic compounds is available but there are several reports concerning their high efficiency in intercepting and retaining general atmospheric particles, especially the smallest ones (Branquinho, 1997, 2001; Branquinho et al., 1999). More than 50% of total chemical content in lichens might be in the particulate form entrapped in the hyphae of the fungi and be retained for very long periods of time. Additionally, the greater values obtained in lichens might be due to their high surface area together with roughness and gelatinous surfaces that may facilitate the interception, uptake and retention of PCDD/Fs bound particles or of PCDD/Fs in the gas phase. Lichen surfaces, having no cuticle, are very efficient in intercepting and retaining particles through atmospheric deposition (Branquinho, 1997; Branquinho et al., 1999); that is the reason why they have been so successfully used for biomonitoring purposes. On the other hand, the greater longevity and slow growth of lichens, compared to plants, could also be a key feature explaining the high content in these organisms. Lichens grow very slowly (from millimetres to few centimetres in a year) because they restrict their metabolism and growth to periods when they are sufficiently hydrated (Armstrong, 1974; Gaio-Oliveira et al., 2004). Moreover, they can live for years or decades and in some cases more than that. Lichens in the arctic environment accumulate radioactivity more than many plants because of their large surface area and long life span (Thomas et al., 1992). In this way, lichens accumulate the pollutants over the period of growth and may integrate the pollution over time. More experiments in this area are needed in order to evaluate which time of exposure to PCDD/Fs is reflected by PCDD/Fs concentrations in lichens.

When compared to other lichen species, the concentrations of PCDD/Fs found in *R. canariensis* are in the same range of magnitude (197.5 to 1218.7 ng kg^{-1} dw) as those found for *Xanthoria parietina* (L.) Th. Fr., collected at the same study region (Augusto et al., 2004); in *X. parietina* values ranged between 73.7 and 1913.3 ng kg^{-1} dw, suggesting that different lichen species probably share key features which lead to high levels of

these compounds in these biomonitoring organisms. The comparison between these two lichen species is published elsewhere (Pereira et al., 2004). These authors found that PCDD/Fs concentrations in *R. canariensis* have more variability and a larger spatial continuity than *X. parietina*, since sill and range of the *R. canariensis* variogram are more or less two-fold those of the *X. parietina* model. In addition, Pereira et al. (2004) found a spatial continuity of 16,000 m for PCDD/Fs in *R. canariensis* collected at the same area of the present study.

Are lichens reflecting the atmospheric PCDD/Fs profile?

Homologues profile

Regarding the mixture of PCDD/Fs homologues present in lichens, the most important contributions to the ΣPCDD/Fs were TCDF and PeCDF, accounting for more than 20% (Figure 2). The contribution of OCDD to the ΣPCDD/Fs was shown to be 4.4% on average, ranging from 2.7% to 7.9%. Based on a systematic literature review compiled by Lohmann and Jones (1998), the average air profile showed a high variability in the relative abundance of the different homologues. For instance, the relative contribution of OCDD to the sum of tetra- to octa-PCDD/F homologue groups differs widely from b10% to N60% and the ratio of PCDDs: PCDFs varies from b0.5% to N2%; the homologues that dominate the air PCDD/F mixture have shown to be hexa- to octa-CDDs and tetra- and penta-CDFs (Lohmann and Jones, 1998).

Although a quite consistent homologue profile of PCDD/Fs in air has been often reported, Lohmann and Jones (1998) have shown that this common view may be incorrect, with large variations in the proportion of different homologues and in the PCDD:PCDF ratio. In this sense, it is more acceptable to compare PCDD/Fs congener profiles rather than homologues patterns, when attempting to evaluate if lichens reflect PCDD/F atmospheric deposition.

Congener profile

In order to discuss whether the selected lichen *R. canariensis* was reflecting the atmospheric PCDD/F profile, we summarized the percentages of contribution of each toxic congener to total PCDD/Fs concentration with a principal component analysis (PCA) (Figures 3A and B). Data from the present study and data obtained from the bibliography for air and soil were used (Table 1). The results are displayed in Table 3 and in Figure 3A and B.

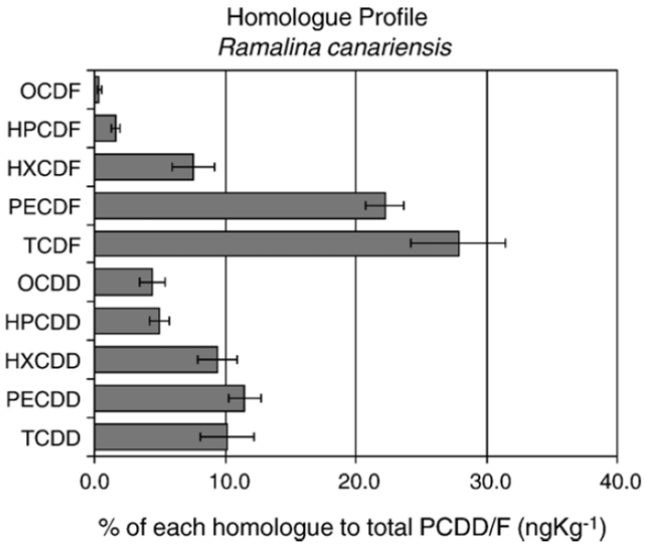

Figure 2. PCDD/Fs homologue profile in the lichen *Ramalina canariensis*. Percentage contribution of each homologue to the ΣPCDD/Fs. n=44. Error bars represent standard deviations.

TABLE 3. Loadings and percentage of explained variance for the first four axes extracted by the PCA analysis of the pollutant data obtained through chemical analysis of the 44 samples of the lichen *Ramalina canariensis* collected in March 2000 in the Setúbal peninsula and the 111 samples of air and the 40 samples of soil extracted from the bibliography. Bold values are significant for p<0.05.

Congeners	Axis 1	Axis 2	Axis 3	Axis 4
2,3,7,8-TeCDD	0.68	−0.20	−0.36	0.01
1,2,3,7,8-PeCDD	**0.89**	0.08	−0.27	−0.19
1,2,3,4,7,8-HxCDD	**0.91**	0.07	−0.26	0.06
1,2,3,6,7,8-HxCDD	**0.91**	0.18	−0.10	0.07
1,2,3,7,8,9-HxCDD	**0.86**	−0.04	−0.33	0.14
1,2,3,4,6,7,8-HpCDD	0.40	0.47	−0.06	0.54
OCDD	**−0.71**	0.59	−0.26	0.05
2,3,7,8-TeCDF	**0.76**	0.23	0.33	−0.22
1,2,3,7,8-PeCDF	**0.88**	0.23	0.14	−0.25
2,3,4,7,8-PeCDF	**0.93**	0.06	0.11	−0.16
1,2,3,4,7,8-HxCDF	**0.82**	−0.04	0.39	0.04
1,2,3,6,7,8-HxCDF	**0.92**	−0.09	0.04	−0.10
1,2,3,7,8,9-HxCDF	0.22	−0.62	−0.58	0.00
2,3,4,6,7,8-HxCDF	0.44	−0.14	0.58	0.52
1,2,3,4,6,7,8-HpCDF	0.05	−0.68	0.33	−0.29
1,2,3,4,7,8,9-HpCDF	0.26	**−0.77**	−0.06	0.42
OCDF	−0.32	**−0.71**	0.06	−0.05
% variance	49.74	16.08	9.01	6.08

Bold values are significant for $P<0.05$.

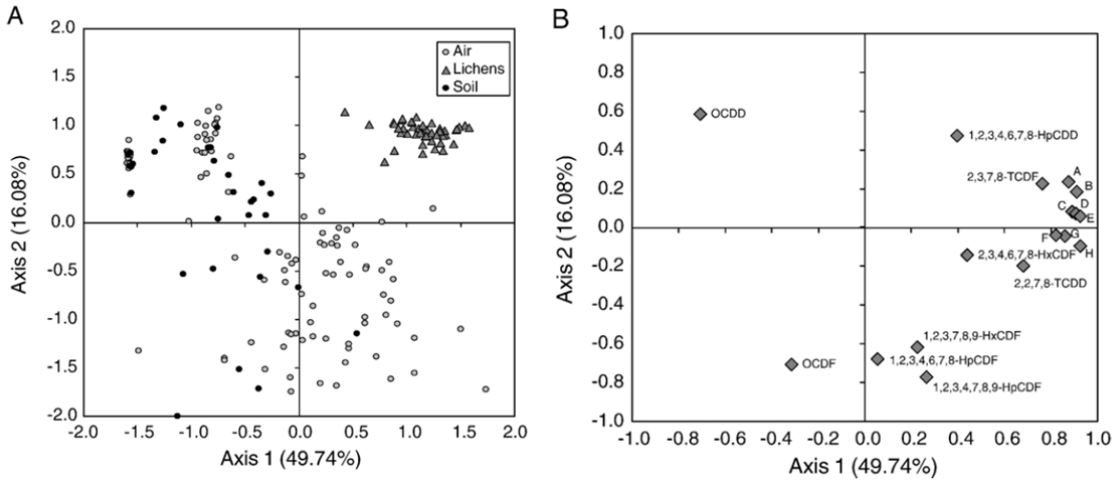

Figure 3. a) Principal component analysis (PCA) between Ramalina canariensis, air and soil PCDD/Fs profiles. Ordination of the lichens, air and soil samples on the two first PCA axes. Each data series is labeled. The first axis accounts for 49.74% of the variance and second axis accounts for 16.08%. b) Principal component analysis (PCA) between R. canariensis, air and soil PCDD/Fs profiles. The first axis accounts for 49.74% of the variance and the second axis accounts for 16.08%. Legend: A-1,2,3,7,8-PeCDF; B-1,2,3,6,7,8-HxCDD; C-1,2,3,7,8-PeCDD; D- 1,2,3,4,7,8-HxCDD; E-2,3,4,7,8-PeCDF; F-1,2,3,4,7,8-HxCDF; G- 1,2,3,7,8,9-HxCDF; H- 1,2,3,6,7,8- HxCDF.

The PCA extracted four factors (or axes), providing a multidimensional model that accounted for 80.9% of the variance (Figures 3A, B and Table 3). The first axis (which explained 49.7% of the variance) associates the congeners 1,2,3,7,8-PeCDD; 1,2,3,4,7,8-HxCDD; 1,2,3,6,7,8-HxCDD; 1,2,3,7,8,9-HxCDD; 2,3,7,8- TeCDF; 1,2,3,7,8-PeCDF; 2,3,4,7,8-PeCDF; 1,2,3,4,7,8-HxCDF and 1,2,3,6,7,8-HxCDF in opposition to OCDD (Figure 3B and Table 3). The second axis (explaining 16.1% of the variance) associates 1,2,3,4,7,8,9- HpCDF and OCDF.

The ordination of the samples on the first two PCA axes (Figure 3A) showed that the first axis clearly separates the PCDD/F profile in lichens from the one in soil samples and associates lichen samples to the majority of the PCDD/F air samples selected for this study. The second axis separates some air samples from lichens and soil. Conjugating both, factor loadings and samples ordination (Figures 3A and B), it can be observed that PCDD/Fs congener pattern in lichens differs from the one found for soils, the main difference lying in the contribution of OCDD to the total toxic PCDD/Fs. As confirmed by

other authors, OCDD dominates the PCDD/Fs profile of soils (Domingo et al., 2001a,b). Soils reflect the accumulation over long periods, and because the more chlorinated PCDD/Fs are retained for longer periods when compared to the less chlorinated PCDD/Fs, the tendency is for an accumulation of OCDD in soils (Domingo et al., 2001a,b).

As shown in Figure 4, the average congener profile, 2,3,4,7,8,-PeCDF makes the single important contribution to the ΣTEQ, accounting for 40%. The minor contributions were from OCDF, 1,2,3,4,7,8,9-HpCDF and OCDD (Figure 4). PCDF contributes more than 50%, while the tetra- and penta-CDD/Fs account for more than 50% of the ΣTEQ (Figure 4).

The former results found in lichens (Figure 4), are in accordance to the general air profile that resulted from the compilation of 26 worldwide air measurements, performed in Europe, America, Japan and Australia (Lohmann and Jones, 1998). The congener profile found in lichens (Figure 4) also was similar to the air profile found by Coutinho et al. (2001) for the atmospheres of Lisbon and Oporto (the most important cities in Portugal), and was also similar to the one found in the atmosphere of the UK (Alcock et al., 2001).

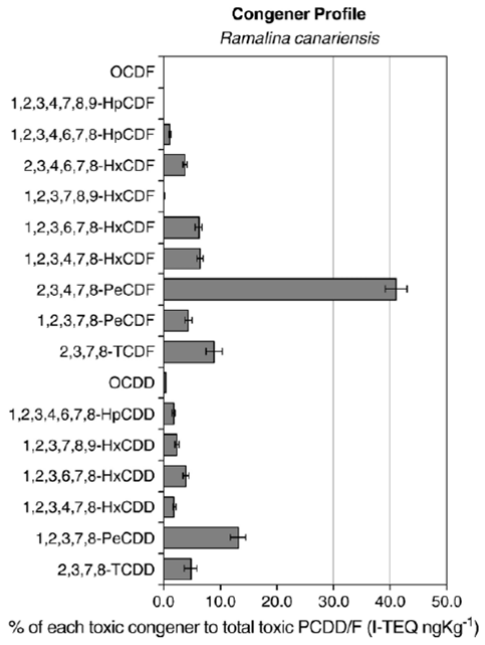

Figure 4. PCDD/Fs congener profile in the lichen *Ramalina canariensis*. Average percentage contribution of each 2,3,7,8-substituted congener to the ΣTEQ; bars are means and lines indicate standard deviations of the 44 samples.

On the contrary, when compared to the typical PCDD/Fs congener profiles detected in Portuguese soils, particularly rural and suburban soils, which are characterized by the contribution of 1,2,3,4,6,7,8-HpCDD accounting for 60% of the ΣTEQ (Coutinho et al., 2002), lichens showed a rather different pattern (Figure 4). Thus, there was no significant contamination from soil of PCDD/Fs in epiphytic lichens of the species *R. canariensis* (Figure 4). This is in accordance to what was already found for heavy metals, where most particles intercepted by lichens, did not reflect contamination from the soil which is absent or minimal (Branquinho, 2001). This is due to the fact that lichens do not have roots, relying only on atmospheric deposition. This conclusion is also supported by the PCA analysis where the PCDD/Fs congener profile in lichens was shown to be different from the one found for soils, the contribution of OCDD to the total toxic PCDD/Fs being the main difference.

Although the air and soil PCDD/Fs measurements were not done at the same sites where lichens were collected, the general air profile obtained was similar to the one in lichens as compared to that of the soils. This means that lichens were not reflecting the typical profile reported for general soil samples and thus were not reflecting particle contamination. This statement could be very useful in biomonitoring studies using lichens to estimate PCDD/Fs atmospheric deposition. The statement that lichens only reflect air pollution is more difficult to demonstrate, when using bibliographic data, due to the substantial differences that PCDD/Fs air profiles have been depending on site location. In future studies, reliable comparisons between lichens and air profiles of the same site should be made. However, a calibration model is not yet possible due to methodological constraints since monitoring stations continually measuring air PCDD/Fs are non-existent to date.

CONCLUSIONS

PCDD/F profiles in *R. canariensis* were more similar to the ones found for air samples rather than the ones found for soil, showing that they are not reflecting soil particle ressuspension or soil vaporization. Compared to other biomonitors (pine needles, fruits, vegetables), lichens have shown to accumulate greater concentrations of PCDD/Fs, meaning that they may provide useful data, especially in areas where levels are below the detection capacity for other monitors. The methodology followed to perform this

study can be applied to other regions of the world, thereby contributing to a better knowledge of PCDD/F levels in ecosystems and their impact on human health.

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2.3 | Lichens as an integrating tool for monitoring PAH atmospheric deposition: A comparison with soil, air and pine needles

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ABSTRACT

The aim of this study was to validate lichens as biomonitors of PAH atmospheric deposition; for that, an inter-comparison between the PAH profile and concentrations intercepted in lichens with those of air, soil and pine needles was performed. The study was conducted in a petro-industrial area and the results showed that PAH profiles in lichens were similar to those of the air and pine needles, but completely different from those of soils. Lichens accumulated higher PAH concentrations when compared to the other environmental compartments and its concentrations were significantly and linearly correlated with concentrations of PAHs in soil; we showed that a translation of the lichen PAHs concentrations into regulatory standards is possible, fulfilling one of the most important requirements of using lichens as biomonitors. With lichens we were then able to characterize the air PAHs profile of urban, petro-industrial and background areas.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are semi-volatile organic compounds, distributed both in the vapor- and particle-phases of the air. PAHs occur naturally in the environment, and are generated by forest fires and volcanic eruptions; however, the largest amount of PAHs is released into the environment by human activities (Edwards, 1983). Anthropogenic PAHs result mainly from pyrolitic processes, especially the incomplete combustion of organic materials during industrial activities, home heating, power generation, incineration and vehicle emissions (ATSDR, 1995; Garban et al., 2002; Dyke et al., 2003; Mastral et al., 2003), and as well as from petroleum cracking and refining in petrochemical industries, and during chemical manufacturing (Kaldor et al., 1984; Mehlman, 1992; Lin et al., 2001; Yang et al., 2002).

In recent years, PAHs have received increased attention in air pollution studies because some of them are highly carcinogenic and mutagenic (IARC, 1983). The US Environmental Protection Agency (EPA) has promulgated 16 unsubstituted PAHs (EPA-PAH) as priority pollutants to be monitored in the environment.

Several methods have been used to assess environmental levels of PAHs, such as soil and sediment, vegetation, food, water and air analyses (Srogi, 2007). The use of biomonitors (living organisms) to evaluate environmental contamination has advantages, as they are easier to sample, allow a long-term monitoring with a large number of sampling sites, and also the simultaneous determination of several pollutants within the same matrix (Wolterbeek, 2002). For air pollution assessment, lichens (symbiotic associations of fungi and algae/cyanobacteria), mosses and pine needles are the most commonly used organisms (Holoubek et al., 2000; Conti and Cecchetti, 2001; Onianwa, 2001; Migaszewski et al., 2002; Landers et al., 2008). Lichens have been used to monitor metals, sulfur, nitrogen, fluoride, radionuclides and a variety of organic compounds, such as dioxins and furans, polychlorinated biphenyls, and substances from organochloride pesticides (Villeneuve et al., 1988; Calamari et al., 1991; Garty, 2000; Augusto et al., 2004, 2007).

Regarding PAHs, there are only a few studies using lichens as biomonitors of these compounds (Herzig, 1989; Owczarek et al., 2001; Migaszewski et al., 2002; Guidotti et al., 2003; Domeño et al., 2006; Blasco et al., 2006, 2007, 2008; Naeth and Wilkinson, 2008; Shukla and Upreti, 2009). The majority of these studies was conducted in natural and forested ecosystems or in urban environments. To date, no comparison of PAH levels and profiles between lichens, soil, pine needles and air has been published for a highly industrialized (mainly petrochemical) and populated area. As PAHs are largely associated with industrial and urban activities, such a comparison will increase our ability to interpret lichen PAH profiles in past and future studies, especially for environmental authorities and decision makers.

The main aims of this study are: to compare PAH concentrations and profiles in lichens with those of soil, air and pine needles in a petro-industrial region; to characterize PAH profiles in urban, industrial and background areas; and to evaluate the possibility of cross-walking PAH concentrations in lichens into concentration units used in regulatory standards.

EXPERIMENTAL SECTION

Sampling for comparison between lichens, soil and pine needles

In January 2008, 34 samples of the species lichen *Parmotrema hypoleucinum* (Steiner) Hale, were collected at a number of sites within the highly industrialized region of Sines, located on the SW coast of continental Portugal, facing the Atlantic Ocean (Figure 1). This region encompasses several important industrial facilities established since the late 1970s: a coal-fired power station, an oil refinery, a chemical plant and, more recently, an industrial landfill as well as many other smaller industries. Moreover, urban development has recently increased.

The lichen *P. hypoleucinum* was selected because it is ubiquitous and tolerates a variety of land-uses, such as urban, industrial, forestry and also background (unmanaged) areas. The collection was made mainly from branches and trunks of *Pinus pinea* L. (umbrella-pine) and *Quercus suber* L. (cork-oak). Samples were packed in brown glass bottles, protected from sunlight and immediately stored at 4°C.

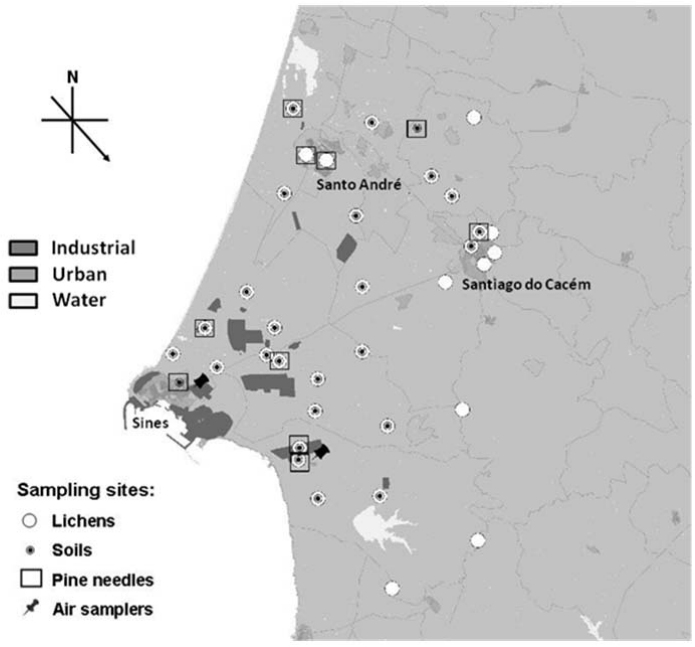

Figure 1. Map of the study area (Sines), 20 × 30 km, showing the sampling sites for lichens (N ¼ 34), soil (N ¼ 26) and pine needles (N ¼ 10), and the location of the two air samplers in the industrial and urban areas. Industrial and urban areas are represented by the darkest colors.

At 26 sites (24 coinciding with lichen sampling points), soil samples were also collected (Figure 1). Samples were taken from the upper 5 cm of soil and placed in polyethylene bags. Once in the laboratory, soil samples were sieved through a 2 mm mesh screen, transferred to glass bottles in order to prevent adsorption by plastic, protected from sunlight and stored at 4 °C. At 10 sites (eight coinciding with lichen sampling points), pine needle samples were also collected. Pine needles from *P. pinea* were selected because there are a considerable number of publications using this plant as biomonitor of toxic organic compounds, and because it is abundant in Europe. Samples were collected from the terminal part of branches, always at the same position on the tree, packed in brown glass bottles, protected from sunlight and immediately stored at 4 °C. All samples were extracted and analyzed for the 16 EPA-PAHs within two months.

Sampling for comparison between lichens and air

Particle-phase samples were collected at two sites (within the urban area of Sines and at the industrial area of Sines) using two high-volume air samplers, which operate with air flows of 66 m3/h and collect particles – PM10 (particles of 10 mm or less diameter) in the urban area and TSP (total suspended particles) in the industrial area – on 20.2 × 25.2 cm cellulose filters (Figure 1). Over a two-month period (February and March 2008), 28 000m^3 of air was sampled at each site and 18 different samples were collected. Each sample corresponded to a 24 h period of continuous sampling. After sampling, the filters were dried, weighed and stored in the dark until analysis. Every 15-day two native lichen samples were collected close to each of the high volume air samplers: *P. hypoleucinum* in the industrial area and *Xanthoria parietina* (L.) Th. Fr. in the urban area. Samples were packed in brown glass bottles, protected from sunlight and immediately stored at 4 °C. All samples were extracted and analyzed for the 16 EPA-PAHs; filters from each 15-day period (corresponding to the lichen samples exposure) were polled together.

Analytical procedure

All PAH analyses took place at the certified laboratory of the Portuguese Environmental Protection Agency (APA). For lichen, soil and pine needle analyses, approximately 2 g of sample was extracted in a Soxhlet with 200 mL of acetonitrile for 24 h. Each group of filters (corresponding to a 15-days sampling) was extracted as a whole. After extraction, all extracts were concentrated by rotary vacuum evaporation and cleaned-up in a florisil

column with 30 mL of acetonitrile as eluting solvent. Subsequently, the extracts were again evaporated and concentrated with a gentle stream of purified N_2 to 1 mL. The samples were analyzed by a high-performance liquid chromatograph (Hewlett Packard), using two columns (Agilent C18 and Phenomenex C18), coupled to an ultraviolet/visible detector (DAD/V–UV) and to an ultraviolet fluorescence detector (FLD). The 16 EPA-PAHs were analyzed, namely: acenaphtylene, naphthalene, fluorene, phenanthrene, fluoranthene, chrysene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, acenaphtene, anthracene, pyrene, benzo[a]pyrene and indeno[1,2,3-cd]pyrene.

Organic matter content of the soil samples was evaluated according to the Loss of Ignition (LOI) method. Samples were dried in order to eliminate water content. Subsequently, they were heated for 2 h at 600 °C and the weight loss was assessed.

Data analysis

Statistical analysis of the results was carried out using the statistical package STATISTICA 8.0 StatSoft Inc. For samples in which a compound was not detected (ND), its concentration was assumed to be the detection limit value. Summary statistics (mean, standard deviation, minimum, maximum and median) were used to characterize PAH concentrations determined within each medium, i.e. lichens, soil and pine needles. Pearsons' linear correlations between lichens and soils were calculated for PAH concentrations and PAH profiles (contribution of each compound to the sum of the 16 EPA-PAHs). A 95% level of significance (P ¼ 0.05) was considered for the results. One-way analysis of variance (ANOVA) followed by Tukey's HSD multiple comparison tests, were conducted to test for significant differences between PAH profiles of different media.

RESULTS AND DISCUSSION

Comparison between lichens, soil and pine needles

The statistical summary of PAH concentrations in lichens, soils and pine needles is presented in Table 1. The total concentrations of 16 PAHs, classified as priority pollutants by US EPA, ranged from 95.5 to 873.8 ng/g (dry weight) in lichens (N ¼ 34), from 27.3 to 769.8 ng/g (dry weight) in soils (N ¼ 26) and from 83.0 to 466.8 ng/ g (dry weight) in pine needles (N ¼ 10). Among the lichen and soil samples collected at the

same sites (N ¼ 24), lichens had higher PAHs concentrations, with a mean value of 238.4 ng/g for the sum of the 16 priority compounds, in contrast with a mean value of 115.6 ng/g in soil samples. Lichen PAH concentrations were above detection levels in most of the analyses (79%), contrasting with soil and pine needles, where PAH concentrations were below detection limits in approximately 50% of the analyses (Table 1). This same result was also observed by Landers et al. (2008).

When compared to soil samples, the highest concentrations measured in lichen samples were unexpected, as soil accumulates PAHs for long periods of time, whereas exposure of lichens is limited to their lifetime. Although lichens tend to accumulate higher concentrations of pollutants than other biomonitors (since they are long-lived organisms), soils are viewed as sinks for organic compounds, and therefore expected to accumulate higher concentrations than biomonitors. The potential of soils for accumulating PAHs mainly depends on their organic matter content and the size of their particles; in this study, the majority of soil samples were of sandy origin, with a mean organic matter content of 2.7%, varying from 0.3 to 12.3%, and with no significant correlation between this variable and the concentration of PAHs (data not shown).

PAHs from a polluted atmosphere are generally transferred to plants through particle-phase deposition on the waxy leaf cuticle or uptake through stomata in the gas-phase (Kipopoulou et al., 1999; Lehndorff and Schwark, 2004). Leaf features (surface, cuticular waxes, hairs, number of stomata, lipids) play an important role in uptake and accumulation of lipophilic compounds such as PAHs (Jouraeva et al., 2002; Howsam et al., 2000). The extent of leaf area exposed to atmosphere, as well as the presence of hairy leaves that increase the leaf surface able to capture particulate from the air, affect the uptake of PAHs through the cuticle (Schreiber and Schönherr, 1992). Lichens are symbiotic organisms formed by fungi and algae/cyanobacteria, and have no roots or cuticle. They absorb all the nutrients and pollutants directly from the atmosphere. Having no cuticle, lichens have no stomata and the whole surface of lichens is exposed to air pollutants and therefore, the total exposed – and intercepting – area is larger than in other biomonitors, such as pine needles, vegetables and fruits. This could explain the higher concentrations detected in lichens when compared to pine needles (Table 1), even considering that the cuticular wax content in pine needles is very high (higher than for other plants), a characteristic which favours their use as biomonitors of organic compounds. On the other hand, Landers et al. (2008) found that the flat-needled conifers

such as *Abies*, *Tsuga* and *Picea* are better accumulators than round needled *Pinus* species.

The PAH concentrations found in *P. hypoleucinum* in the present study are within the same range of those found in other studies in which concentrations range from 25 ng/g in *X. parietina* and 6420 ng/g in *Evernia prunastri* (Migaszewski et al., 2002; Guidotti et al., 2003; Domeño et al., 2006; Blasco et al., 2006, 2007, 2008).

Additionally, in our lichen samples the dominant compounds were phenanthrene, pyrene and fluoranthene (Table 1), which correspond to the dominant compounds found in lichens by other authors (Migaszewski et al., 2002; Guidotti et al., 2003; Domeño et al., 2006; Blasco et al., 2006, 2007, 2008).

Regarding PAHs in soil (Table 1), the concentrations were generally lower than those reported in other studies from different regions and countries, which range between 49.4 ng/g for control unburnt soil of Korea to 47 870 ng/g for soils near an oil refinery after the Kosovo war in Serbia and Montenegro (Kim et al., 2003; Skrbic and Miljevic, 2002). In Tarragona County, Spain, Nadal et al. (2004) found concentrations ranging from 112 to 1002 ng/g (dry weight) for soil samples of unpolluted sites and collected near chemical industries, respectively.

According to Maliszewska-Kordybach (1996), soils can be classified as not contaminated if PAHconcentrations are under 200 ng/g; weakly contaminated if concentrations are between 200 and 600 ng/g; contaminated if they range from 600 to 1000 ng/g; and heavily contaminated if concentrations are over 1000 ng/g. The majority of our soil samples was under 200 ng/g and thus can be classified as being not contaminated; two samples had values between 200 and 600 ng/g, being weakly contaminated and only one sample presented concentrations over 600 ng/g, being contaminated. This classification was derived from determinations of PAH concentrations in European soils, as well as from an estimation of risks of human exposure (Paterson and McKay, 1989). According to this classification, the region studied in the present work can be generally considered as not contaminated, presenting some contamination spots.

The most concentrated PAHs in lichens and pine needles were phenanthrene, pyrene, fluoranthene and naphthalene; in soil samples the most concentrated were naphthalene, phenanthrene and pyrene (Table 1). These results are in agreement with the data from the Portuguese Environmental Protection Agency for PAHs measurements in the study

area regarding the wet deposition for the same period of our study, except for fluoranthene (APA, 2008). The most concentrated compounds found in wet deposition were naphthalene, phenanthrene and pyrene, whereas in dry deposition were naphthalene, fluorene, phenanthrene and pyrene (APA, 2008).

TABLE 1. Statistical summary of PAH concentrations (ng/g) in lichens collected at 34 sampling sites, in soil collected at 26 sampling sites and in pine needles collected at 10 sampling sites. SD, standard deviation; Min, minimum; Max, maximum; Med, median; N, number of samples over detection limits. Mean, standard deviations, minimums, maximums and medians were calculated using zero for under detection limit values.

	Lichens						Soil						Pine needles					
	Mean	SD	Min	Max	Med	*N*	Mean	SD	Min	Max	Med	*N*	Mean	SD	Min	Max	Med	*N*
Acenaphtylene	1.0	0.0	1.0	1.0	1.0	0	7.1	8.8	5.0	49.3	5.0	2	5.0	0.0	5.0	5.0	5.0	0
Naphthalene	24.4	29.6	7.7	156.3	16.3	34	23.1	32.7	2.0	161.3	16.4	26	18.6	13.5	1.0	34.4	22.1	9
Fluorene	5.8	3.7	2.8	19.0	4.5	34	4.0	2.4	2.6	14.2	3.2	26	15.1	7.0	7.0	28.0	16.2	10
Phenanthrene	62.0	36.6	27.7	202.2	50.8	34	20.0	31.1	3.1	149.5	9.9	26	67.7	46.8	25.6	172.2	47.4	10
Fluoranthene	50.2	34.4	17.6	173.9	37.3	34	8.0	11.8	1.0	51.8	3.7	24	24.0	22.7	8.2	84.6	17.4	10
Chrysene	12.7	7.2	3.9	30.3	10.3	34	6.0	11.7	1.0	54.0	1.4	19	9.6	6.8	1.4	21.3	8.5	10
Benzo[a]anthracene	11.2	10.8	2.9	54.0	8.6	34	3.8	7.3	0.9	29.1	1.0	14	6.6	5.0	1.0	13.3	4.9	10
Benzo[b]fluoranthene	8.2	7.4	2.8	41.3	6.8	34	6.8	14.0	1.0	64.7	1.2	15	4.4	2.5	0.0	7.3	4.7	9
Benzo[k]fluoranthene	3.2	3.6	0.0	19.4	2.3	34	3.3	5.9	1.0	22.6	1.0	10	1.0	0.0	1.0	1.1	1.0	1
Dibenzo[a,h]anthracene	1.1	0.6	1.0	4.5	1.0	1	1.0	0.1	1.0	1.5	1.0	1	1.0	0.0	1.0	1.0	1.0	0
Benzo[g,h,i]perylene	5.3	7.8	1.0	44.4	3.1	32	6.5	14.1	1.0	67.9	1.0	13	1.0	0.0	1.0	1.0	1.0	0
Acenaphtene	1.9	1.3	0.9	7.0	1.2	25	1.0	0.3	0.0	2.2	1.0	2	3.5	5.6	1.0	19.0	1.0	4
Anthracene	3.8	3.6	1.0	12.3	1.8	27	1.1	0.5	1.0	3.4	1.0	1	4.9	5.8	1.0	20.7	4.2	8
Pyrene	52.1	53.0	1.0	208.7	32.0	33	15.2	30.6	1.0	141.5	6.2	24	20.7	25.0	1.0	83.4	14.0	7
Benzo[a]pyrene	3.7	5.4	1.0	26.0	1.6	24	1.6	2.5	1.0	13.7	1.0	22	1.1	0.3	1.0	2.0	1.0	1
Indeno[1,2,3-cd]pyrene	2.2	3.9	0.0	21.7	1.0	13	3.9	6.6	1.0	28.5	1.0	9	1.2	0.7	1.0	3.2	1.0	1
16 EPA-PAH	**249.0**	**171.3**	**95.5**	**873.8**	**195.6**	**34**	**112.3**	**161.9**	**27.3**	**769.8**	**64.4**	**26**	**185.4**	**113.5**	**83.0**	**466.8**	**142.7**	**10**

The PAH profiles (contribution of each compound to the sum of the 16 EPA-PAHs) in lichens, soils and pine needles collected at the same sites are depicted in Figure 2.

The lowest-ring PAHs (2-ring) were dominant in soils, whereas 4-ring PAHs were the dominant group in lichens, and 3-ring PAHs were most important in pine needles. Blasco et al. (2006, 2008) found that PAH profiles in the lichens *Parmelia sulcata* and *E. prunastri* were dominated by 3-ring PAHs, whereas the lowest contribution was from 6-ring PAHs. PAHs with two and three rings in their structure are partially or totally present in the vapor phase of the atmosphere, 4-ring PAHs are present in both vapor and particulate matter, and PAHs of higher molecular weight (five and six rings) are generally associated with particulate matter (Guidotti et al., 2003). Lichens are very efficient in absorbing nutrients (and also pollutants) from the vapor phase, and this could explain the higher contribution of low ring PAHs to the profiles found in lichens.

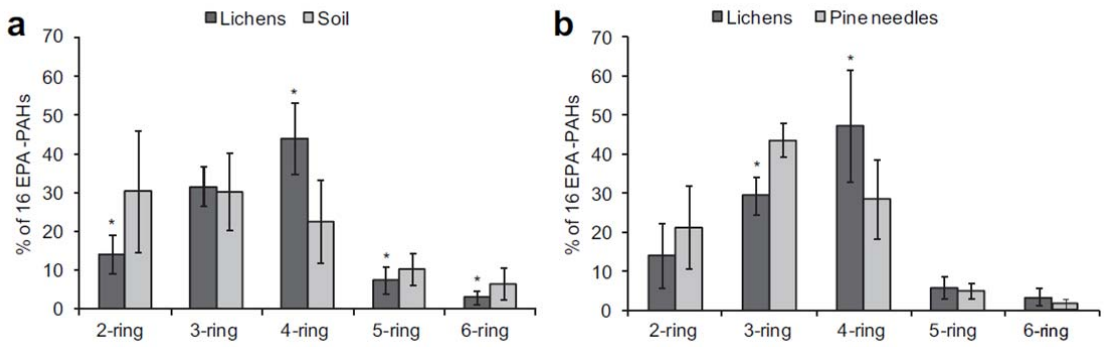

Figure 2. PAH profiles in lichen and soil samples collected at the same 24 sites (a) and in lichens and pine needles collected at the same 8 sampling sites (b). Results are given as percentages of the sum of concentrations of the total 16 EPA-PAHs. Bars represent standard deviations. Tukey's post hoc test: asterisks indicate significant differences between lichens and soil or lichens and pine needles ($P<0.05$).

TABLE 2. Pearson's correlation between lichens and soil for PAH concentrations and PAH profiles. Correlations in bold are significant at $P<0.05$. N=24. The correlation coefficient and the significance level for each correlation between each PAH compound concentration in lichens and in soil are shown, as well as for each correlation between the contribution of each PAH to the sum of the 16 EPA-PAH (profiles) in lichens and in soil.

	Lichens *vs.* soil			
	Concentrations		Profiles	
	Correlation coefficient	Significance level	Correlation coefficient	Significance level
Acenaphtylene	−0.0521	0.809	−0.0361	0.867
Naphthalene	**0.9401**	0.000	0.3716	0.074
Fluorene	0.3899	0.060	0.0608	0.778
Phenanthrene	**0.8951**	0.000	0.3496	0.094
Fluoranthene	**0.7105**	0.000	0.2956	0.161
Chrysene	**0.7811**	0.000	0.2291	0.282
Benzo[a]anthracene	**0.6546**	0.001	−0.2583	0.223
Benzo[b]fluoranthene	**0.8873**	0.000	0.3203	0.127
Benzo[k]fluoranthene	**0.7123**	0.000	0.1530	0.475
Dibenzo[a,h]anthracene	–	–	0.2568	0.226
Benzo[g,h,i]perylene	**0.9195**	0.000	**0.4423**	0.030
Acenaphtene	0.3181	0.130	−0.2310	0.277
Anthracene	0.3329	0.112	0.2025	0.343
Pyrene	**0.8759**	0.000	0.2379	0.263
Benzo[a]pyrene	0.3973	0.055	−0.2674	0.206
Indeno[1,2,3-cd]pyrene	**0.4915**	0.015	0.1619	0.450
2-ring PAHs	**0.9408**	0.000	0.3507	0.093
3-ring PAHs	**0.8641**	0.000	**0.6172**	0.001
4-ring PAHs	**0.9013**	0.000	0.3653	0.079
5-ring PAHs	**0.8565**	0.000	0.0184	0.932
6-ring PAHs	**0.8434**	0.000	0.3286	0.117
16 EPA-PAH	**0.9457**	0.000	–	–

Regarding PAH profiles (contribution of each compound to the sum of the 16 EPA-PAHs), there were no significant correlations between lichens and soil for the majority of the compounds, the exceptions being the benzo[g,h,i]perylene (Table 2). This means that the PAH profile detected in lichens is not correlated with the PAH profile detected in soil.

Figure 3 shows the PAH profiles for lichens and soil collected at sites where three different land-uses dominate – urban, industrial and background. As background, a coastal forestry area situated at the north of the main industrial facilities was considered; the prevailing winds are from N–NW and the background area is therefore dominated by oceanic winds. Lichens and soil in background areas showed significant differences for the 4-ring PAHs (as showed by the Tukey's post hoc test, Figure 3); however, in urban and industrial areas, where the atmospheric deposition of PAHs is higher, the profiles tend to be similar, showing that both lichens and soil are reflecting the same ambient pollution at high contaminated sites (Figure 3).

Although the PAH profiles of lichens and soils at all sampling sites were uncorrelated, PAH concentrations, on the other hand, were significantly correlated for the majority of compounds, except for acenaphtylene, fluorene, acenaphtene, anthracene and benzo-a-pyrene (Table 2). In order to intercalibrate lichens and soil, a bi-plot was drawn for the concentration of the sum of the 16 EPA-PAHs (Figure 4).

Although these monitors display different profiles for PAH, a correlation between the total concentrations of PAHs was found for the most highly contaminated samples. The PAH concentrations in the majority of soil samples were within background value ranges; in contrast, lichens were more sensitive, displaying a wide range of PAH values. To develop a consistent and robust correlation model between lichens and soil more samples from contaminated sites would be necessary. However, our results clearly show that the translation of lichen PAH concentrations into soil concentrations seems to be possible.

Soils have European classification based on their PAH concentration; translating the concentrations determined in lichens into concentrations measured in soils will allow classification of the location according to its PAH contamination.

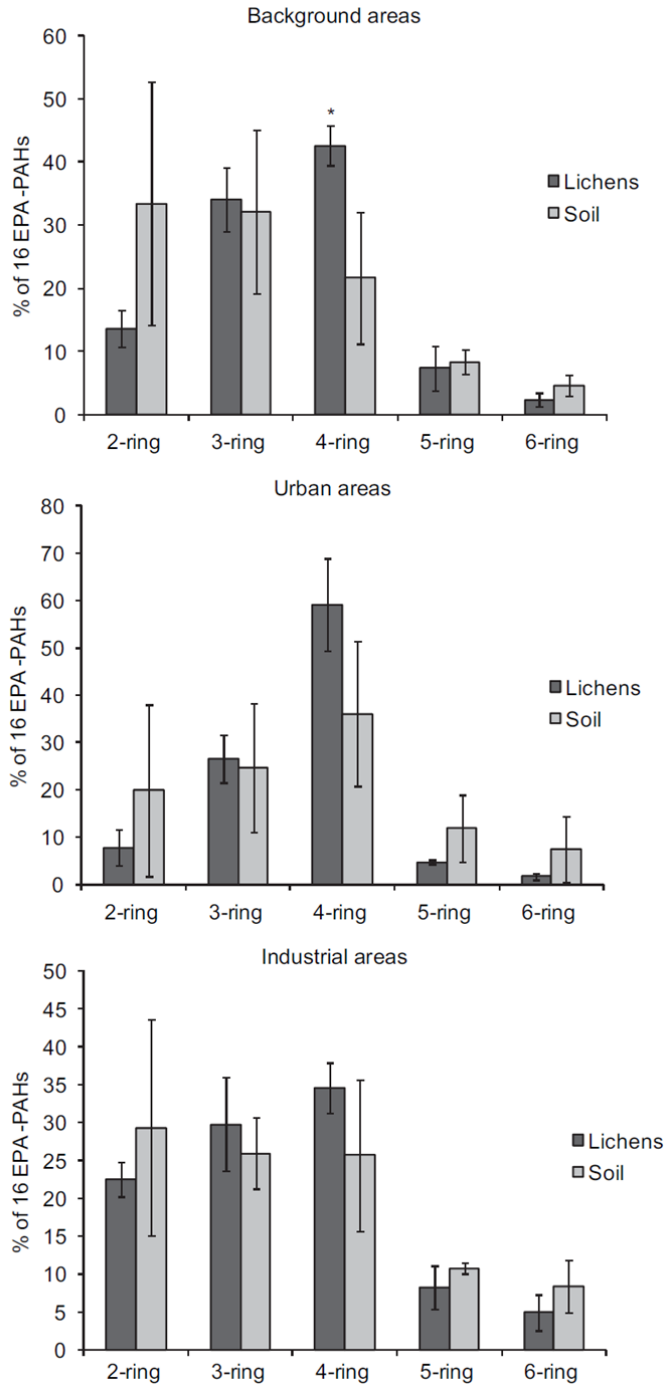

Figure 3. PAH profiles in lichens and soils collected at three different sites subjected to different types and levels of pollution – urban, industrial and background. Results are given as percentages of the sum of concentrations of the total 16 EPA-PAHs. Bars represent standard deviations. Tukey's post hoc test: asterisks indicate significant differences between lichens and soil ($P<0.05$).

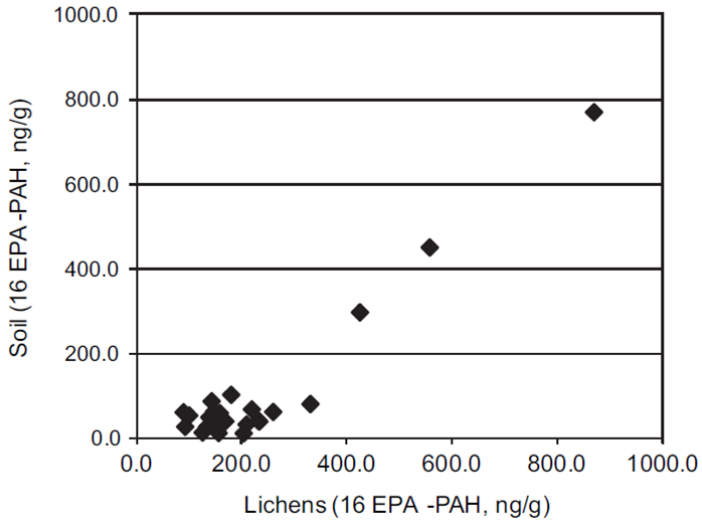

Figure 4. Bi-plot for the sum of 16 EPA-PAH concentrations in lichens and in soil. N=24.

Lichens in relation to air samples

PAH concentrations for the sum of the 16 EPA-PAHs in air ranged between 23.8 and 40.1 ng/m^3 at the industrial site and between 11.0 and 18.9 ng/m^3 at the urban site. These values are within the same order of magnitude as atmospheric PAH concentrations in PM_{10} particulates at different sites in the world (Srogi, 2007). Lichens ranged between 442.6 and 562.0 ng/g for *P. hypoleucinum* samples collected at the industrial site and between 167.3 and 256.3 ng/g for *X. parietina* samples collected at the urban site, in agreement with the levels found in the air samplers.

Figure 5 shows the PAH profiles found for air samples (particle phase) and for lichens collected at the two sites.

Both profiles are dominated by 4-ring PAHs. Lichens showed higher contributions of low molecular PAHs (2- and 3-ring PAHs), whereas air presented higher contributions of high molecular PAHs (5- and 6-ring PAHs). These air profiles are consistent with other studies where authors analyzed PAHs in the particle-phase and found similar profiles (Blasco et al., 2006, 2008). High proportions of heavy PAHs are often found in particles (Feilberg and Nielson, 2000; Lightly et al., 2000). The low molecular-weight PAHs are predominantly present in the vapor phase, and can be monitored using a sorbent sponge coupled to the high-volume air sampler. Lichens seem to intercept both the particle and

the vapor phase of air, suggesting their potential as integrating tools for monitoring PAH deposition.

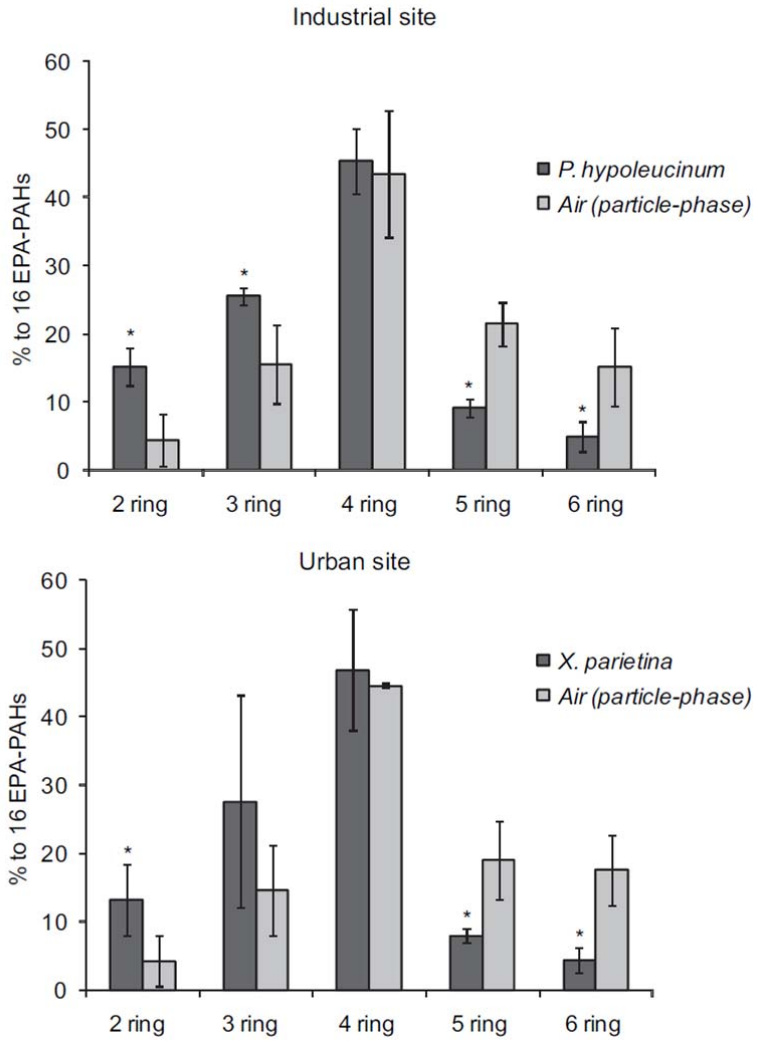

Figure 5. PAH concentration patterns in lichens and in air (particle-phase) samples collected at the same sampling sites. Results are given as percentages of the sum of concentrations of the total 16 EPA-PAHs. Bars represent standard deviations. Tukey's post hoc test: asterisks indicate significant differences between lichens air ($P<0.05$).

CONCLUSIONS

In conclusion it was found that lichens accumulate higher concentrations of PAHs than soil and pine needles, and that they also allow detection for the majority of the 16 EPA-PAH. The average PAH profiles for lichens and soils are distinct in background areas, but tend to be similar in urban and industrial areas, where atmospheric deposition of PAHs

is higher. Nevertheless, although profiles were not correlated, PAH concentrations in lichens were correlated with those found for soils in contaminated sites, showing the potential for the translation of lichen values into the European classification of PAH contaminated sites, fulfilling in this way one of the most important requirements of using lichens as biomonitors.

ACKNOWLEDGMENTS

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2.4 | A step towards the use of biomonitors as estimators of atmospheric PAHs for regulatory purposes

Submitted

ABSTRACT

One of the main drawbacks of using lichens to monitor atmospheric PAHs has been reported as the impossibility to translate PAH values in lichens into the atmospheric equivalents ones, in order to use this information for regulatory purposes. In this work, for the first time, PAH concentrations in lichens were calibrated against PAH concentrations in a conventional active sampler in an outdoor environment for a nine-month span. Results showed significant positive correlations between HMW-PAHs, Σ16 EPA-PAHs, and BaP equivalent concentrations in lichens and those in particulate-phase of air, especially from the 45-days prior to lichen collection. Concentrations of Σ16 EPA-PAHs in lichens and air showed a seasonal variation, with highest values during winter and lowest values during summer. We suggested that air temperature and sunshine radiation might be the main factors responsible for the seasonal variation in PAH concentrations in air and lichens. For regulatory purposes, a calibration between lichens and air was obtained for BaP, for the Σ16 EPA-PAHs, and for BaP equivalent concentrations. These calibrations allow integrating lichens more broadly into PAH monitoring schemes.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds composed of two or more fused aromatic rings. They have received increased attention in recent decades in air pollution studies due to their carcinogenicity and mutagenicity (USEPA, 1993). Although hundreds of PAH compounds exist in the environment, only 16 of them are classified as priority pollutants by the US Environmental Protection Agency (USEPA, 2003). Due to their mobility, persistence, tendency to bioaccumulation, and toxic effects on human health, PAHs have been included in the Convention on Long Range Transboundary Air Pollution Protocol on Persistent Organic Pollutants (Council Decision 2004/259/EC). They form mainly through incomplete combustion of fossil fuels and biomass (pyrogenic origin) and spills of petroleum derivatives (petrogenic origin). Sources of PAHs in the atmosphere include automobiles, resuspended soils, refineries, and power plants (Yang et al., 2002; Dyke et al., 2003). Strategies to monitor these compounds in the environment comprise air, soil and water monitoring, and more recently food and biomonitoring tools (Srogi, 2007; Augusto et al., 2010, 2011). Air

monitoring is usually made measuring PAHs present in the particulate- and vapor-phases of atmosphere; though PAHs present in vapor-phase are important, PAHs in the particulate-phase are considered to have greatest health impact (Smith and Harrison, 1998). Directive 2004/107/EC from European Union Law states that the 5-ring compound benzo[a]pyrene should be measured in the PM_{10} fraction of the particulate-phase of air, as this compound is considered an indicator of the remaining PAHs (Directive 2004/107/EC). The maximum admissible level for this compound in the ambient air has been fixed at $1ng/m^3$ – average value for one year of measurements (Directive 2004/107/EC). However, PAH air monitoring has several constraints, particularly: i) reflects a short-term indicator that varies considerably in space and time; ii) active air samplers required to monitor PAHs in ambient air are expensive and require physical installation and energy supply, which means less samplers can actually be installed to cover all territory; iii) doesn't provide information on the long-term impact on ecosystems (including, vegetation, animals, and humans). These constraints are an important weakness in environmental health studies. PAH monitoring data measured in conventional active air samplers are in general considered to be representative of a large area (due to the scarcity of samplers) and it is assumed that populations living in large areas are all exposed to the same levels of PAHs. In order to assess human exposure to these compounds it is important to be able to distinguish between exposed and control populations. For that, it is essential to develop tools that are able to assess the spatial deposition of PAHs with high spatial resolution. Biomonitoring methods (use of living organisms) have been developed during the last decades to fulfill this gap (Srogi, 2007). Furthermore, Directive 2004/107/EC itself allows the use of biomonitoring tools to assess spatial deposition of PAHs. Within biomonitors, lichens (symbiosis between fungi and algae and/ or cyanobacteria) are one of the most used organisms to monitor atmospheric deposition of several air pollutants (Branquinho, 2001). Lichens are long-lived biomonitors, and thus they are long term integrators of the atmospheric pollution deposition. This characteristic is of crucial importance for evaluating human exposure to pollutants such as PAHs; time integration of these compounds allows relating low levels of pollutants with long-term chronic effects on health (Augusto et al., 2007). Additionally, there are studies showing that lichens can be used to monitor PAH atmospheric deposition (Augusto et el., 2009, 2010; Guidotti et al., 2003; Domeño et al., 2006; Blasco et al., 2006, 2007, 2008; Shukla and Upreti, 2009). Blasco et al. (2008) used lichens and found that the road traffic was the

main source of PAHs in the Pyrenees Mountains region. These authors found that PAHs in lichens reflected the atmospheric particulates when they studied the PAH pollution caused by vehicle emissions in a tunnel but they did not made a calibration (Blasco et al., 2006). When comparing lichens to soil and air, it was shown that the profile of PAHs in lichens was substantially different from that of the soil but similar to that of the air; it was also revealed that lichens intercept PAHs both from the vapor- and particulate-phases of air (Augusto et al., 2010). More recently, using spatial models of PAHs in lichens it was possible to fingerprinting multiple sources of atmospheric PAHs in a regional area (Augusto et al., 2009). Thus, lichens seem to be an excellent candidate for biomonitoring PAHs in the atmosphere.

However, one of the main drawbacks of using lichens to monitor atmospheric PAHs has been reported as the impossibility to translate PAH values in lichens into the atmospheric equivalents ones, in order to use this information for regulatory purposes. Thus, the main aim of this study was to, for the first time, calibrate PAH concentrations in lichens against PAH concentrations measured in a conventional active sampler in an outdoor environment to be able to use the advantages of lichens as biomonitors and be able to use them more broadly for monitoring PAH pollution in the atmosphere.

EXPERIMENTAL SECTION

Sampling

This study was developed in the highly industrialized region of Sines, located on the SW coast of continental Portugal (Europe) facing the Atlantic Ocean. This region encompasses several important industrial facilities established in the late 1970s: a coal-fired power station, an oil refinery, a chemical plant, an industrial waste water treatment plant; and an industrial landfill as well as many other smaller industrial plants, based primarily on the processing of oil products. Particulate-phase samples were collected using a high-volume air sampler located in the south limit of the industrial area, which collects particles –TSP (total suspended particles) – on 20.2×25.2 cm cellulose filters. Over a nine-month period (January to September 2008), 143 568 m^3 of air was sampled and 90 different samples were collected (number of filter samples collected during each month is displayed in Table 1). Each sample corresponded to a 24 h period of continuous sampling. The gap between sampling periods was 48 hours. After

sampling, filters were dried, weighed (to assess concentration of particles) and stored in the dark until analysis. Native lichens of the species *Parmotrema hypoleucinum* (Steiner) Hale were collected close to the high volume air sampler, every 15 days from February to May and every 30 days from June to September 2008 – a total of 13 lichen samples were collected. The collection was made from branches and trunks of *Quercus suber* L. (cork-oak). Samples were packed in brown glass bottles, protected from sunlight and immediately stored at 4 ºC. All samples were extracted and analyzed for the 16 EPA-PAHs; filters from each 15 days (from January to September) were polled together – a total of 19 sample groups was obtained.

PAH analysis

All PAH analyses took place at the certified laboratory of the Portuguese Environmental Protection Agency (APA). For lichens, approximately 2 g of sample was placed in a Soxhlet with 200 mL of acetonitrile (HPLC grade) for 24 h. Each group of filters (corresponding to a 15-days sampling) was extracted as a whole. After extraction of the PAHs from the samples, extracts were concentrated by rotary vacuum evaporation and cleaned-up in a florisil column with 30 mL of acetonitrile as eluting solvent. Subsequently, extracts were again evaporated and concentrated with a gentle stream of purified N_2 to 1 mL. The samples were analyzed by high performance liquid chromatography (Hewlett-Packard), using two columns (Agilent C18 and Phenomenex C18), coupled to an ultraviolet fluorescence detector (FLD) and to an ultraviolet/visible detector (DAD/V-UV). The acetonitrile/water gradient profile was 50:50 for 5 min, 60:40 over 15 min, 90:10 for 4 min, 80:20 during 6 min, 90:10 for 10 min, and finally 100:0 over 5 min at a flow rate of 1 mL/min. Column temperature was kept at 28 °C.

The 16 priority EPA-PAHs were analyzed, namely: acenaphtylene (ACPHY), naphthalene (NAPH), fluorene (FLU), phenanthrene (PHEN), fluoranthene (FA), chrysene (CHR), benzo[a]anthracene (BaA), benzo[b]fluoranthene (BbFA), benzo[k]fluoranthene (BkFA), dibenzo[a,h]anthracene (DBahA), benzo[g,h,i]perylene (BghiP), acenaphtene (ACPH), anthracene (ANTH), pyrene (PY), benzo[a]pyrene (BaP), and indeno[1,2,3-cd]pyrene (IP). The majority of compounds presented concentrations above detection limit, except ACPHY and DBahA which presented values under detection limit for all lichen samples. For samples in which a compound was not detected (ND), its concentration was assumed to be the detection limit value. PAH standards of Ultrascientific with an uncertainty of 5% were used.

BaP equivalent concentrations

The carcinogenic risk of a PAH mixture is often expressed by its benzo[a]pyrene equivalent concentration (BaPeq). Based on the carcinogenic potency of each other individual PAH relative to that of BaP (Toxic Equivalent Factors, TEFs), the carcinogenic potency of each PAH in the mixture is expressed by its BaPeq. There are different TEFs developed by different agencies and scientists (USEPA 1993; MOE, 1997; CEPA, 1994; cal EPA, 1003; Nisbet and LaGoy, 1992). In our study we've adopted the TEFs developed by Nisbet and LaGoy (1992), as these values are most commonly used while assessing the carcinogenic potency of PAH mixtures (Tsai et al., 2001). In this way, for each sample it was calculated the total carcinogenic potency through the sum of BaPeq concentrations calculated for each of the sixteen compounds.

Meteorological variables

Temperature data during the studied time span were collected the closest weather station to the sampling location. Seasons were defined by solstices and equinoxes: winter (21 December -19 March), spring (20 March – 20 June), summer (21 June – 20 September), and autumn (21 September – 20 December).

Statistical analysis

Descriptive statistics (mean, standard deviation, minimum and maximum) were used to characterize PAH concentrations determined within lichens and particulate-phase of air. Pearson's linear correlations (performed after checking normality of data) between lichens and particulate-phase of air (correspondent to the 15, 30, 45 and 60 days prior to lichen sampling) were calculated for PAH concentrations - for low molecular weight PAHs (LMW-PAHs) (consisting of 2- and 3-ring PAHs), for high molecular weight PAHs (HMW-PAHs) (consisting of 4-, 5- and 6-ring PAHs), for the sum of the 16 EPA-PAHs, and for BaP equivalent concentrations (BaPeq.). Pearson's linear coefficients were also calculated for the correlation between PAH concentrations (in lichens and particulate-phase of air) and the average temperature for 45 days prior to lichen sampling and for the average and maximum temperatures from the one to seven days prior to lichen sampling. Results were considered statistically significant if the p-value was less than 0.05.

RESULTS

PAH in lichens and in particulate-phase of air

In this work we found that concentrations of 16 EPA-PAHs in lichens ranged from 58 ng PAH/g to 556 ng PAH/g. The greatest concentrations were found during the coldest winter months (February and March), while the lowest were found during the warmest summer months (July, August and September) (Figure 1). Concentrations of the 16 EPA-PAHs in the particulate-phase of air varied between 0.077 ng PAH/m^3 and 1.156 ng PAH/m^3; as in lichens, the greatest values were found during winter and the lowest values during summer (Figure 1).

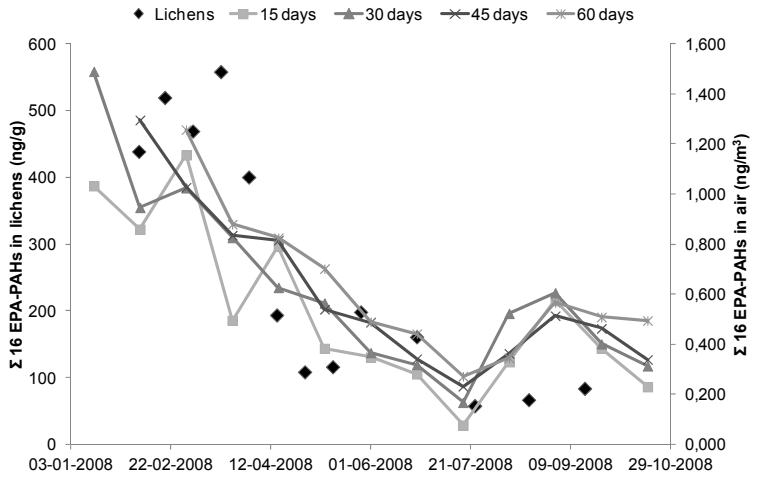

Figure 1. Temporal variation of the sum of 16 EPA-PAHs in lichens and in the particulate-phase of air measured during the 15, 30, 45 and 60 days prior to lichen collection dates.

The overall TSP concentration collected during the time span of the study was 50.4 µg/m^3 (Table 1); lowest concentrations were observed during winter, while greatest concentrations were observed during summer. Regarding BaP concentrations, in particulate-phase of air ranged from 0.0040 to 0.1143 ng/m^3, while in lichens ranged from 2.67 to 16.96 ng/g.

Regarding PAH ring profile, we found that profile in lichens was dominated by 2-, 3- and 4-ring PAHs, while in the particulate-phase of air it was dominated by 4-, 5- and 6-ring PAHs (Figure 2).

TABLE 1. Total Suspended Particulate (TSP) concentration measured in filters collected in each month during the sampling period – from January 2008 to September 2008. Number of filters collected during each month, as well as average monthly temperatures and minimum and maximum temperatures are also displayed.

	TSP (µg/m3)	Number of filters	Average temperature (° C)	Minimum temperature (° C)	Maximum temperature (° C)
Jan	32.7	10	13.0	7.5	18.8
Feb	28.6	9	13.2	5.5	19.4
Mar	47.6	10	12.6	5.2	21.6
Apr	43.9	10	15.2	8.7	28.3
May	46.7	11	15.8	9.8	23.0
Jun	80.9	10	19.1	12.1	28.9
Jul	52.6	10	19.1	13.4	25.4
Aug	74.0	10	19.3	13.7	26.8
Sep	44.5	10	19.0	12.9	27.1
Total	50.4	90	16.3	9.9	24.4

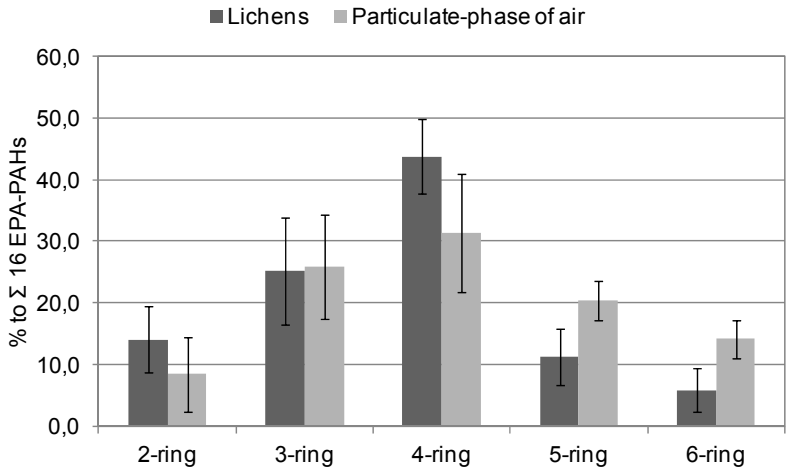

Figure 2. PAH ring profile in lichens and in the particulate-phase of air measured during the 45 days prior to lichen sampling dates (N=12). Bars represent standard deviations.

Correlations between PAHs in lichens and in particulate-phase of air

Pearson's correlations between PAH concentrations in lichens and in the particulate-phase of air measured during 15, 30, 45 and 60 days prior to lichen sampling dates showed positive significant correlations for the HMW-PAHs, for the sum of the 16 EPA-PAHs and for the total BaP equivalent concentration (Table 2). The greatest correlation coefficients were found for correlations between PAHs in lichens and in particulate-

phase of air from the 45-days prior to lichen collection (Table 2). No significant correlation was obtained between LMW-PAHs in lichens and in particulate-phase of air (Table 2).

Influence of temperature

Significant negative correlations were found between PAH concentrations in lichens and temperature for all PAH groups – LMW-, HMW-PAHs and the sum of 16 EPA-PAHs - without exception, while a significant positive correlation was found between PAH concentrations in the particulate-phase of air and temperature but only for LMW-PAHs (Table 3).

TABLE 2. Significant Pearson's coefficients for correlations between PAH concentrations in lichens and in the particulate-phase of air measured during 15 (N=13), 30 (N=13), 45 (N=12) and 60 (N=11) days prior to lichen sampling dates. Results are displayed for low molecular weight (LMW) PAHs, consisting of 2- and 3-ring PAHs; high molecular weight (HMW) PAHs, consisting of 4-, 5- and 6-ring PAHs; sum of the 16 priority EPA-PAHs; and for BaP equivalent concentrations (BaPeq.). Bold: significant < 0.05.

	air from 15 days N=13		air from 30 days N=13		air from 45 days N=12		air from 60 days N=11	
LMW-PAHs	-0.0283	p=0.027	-0.1981	p=0.516	-0.2098	p=0.513	-0.4102	p=0.210
HMW-PAHs	**0.8055**	p=0.001	**0.8190**	p=0.001	**0.9002**	p=0.000	**0.8419**	p=0.001
Σ16 EPA-PAHs	**0.7095**	p=0.007	**0.7449**	p=0.003	**0.8517**	p=0.000	**0.7718**	p=0.005
BaPeq.	0.5192	p=0.069	**0.5547**	p=0.049	**0.7975**	p=0.002	**0.6838**	p=0.020

TABLE 3. Pearson's correlation coefficients for LMW-, HMW-PAHs and Σ16 EPA-PAHs in lichens and in particulate-phase of air measured during the 45 days prior to lichen sampling dates, and the average temperature of the same time spans. N=12. Bold: significant < 0.05.

Relation with average temperature for 45 days			
Lichens	**LMW-PAHs**	**-0.7060**	p=0.010
	HMW-PAHs	**-0.6236**	p=0.030
	Σ16 EPA-PAHs	**-0.6634**	p=0.019
Air from 45 days	**LMW-PAHs**	**0.6440**	p=0.024
	HMW-PAHs	-0.5376	p=0.071
	Σ16 EPA-PAHs	-0.4344	p=0.158

Calibration of lichens against particulate-phase of air

Figure 3 illustrates the linear relationships between the concentrations of BaP (which is used as marker for the carcinogenic risk of PAHs in ambient air, as per Directive 2004/107/EC) (10), the sum of the 16 EPA-PAHs and the sum of the BaP equivalent concentrations in lichens and in the particulate-phase of air measured during 45 days prior to lichen collection dates. Forty five days was the retroactive time span selected for calibration, as it showed greatest Pearson's correlation coefficients for most of PAHs (Table 2). The coefficient of determination for BaP was as high as 0.588; for the sum of the 16 EPA-PAHs and the sum of the BaP equivalent concentrations, coefficients were as high as 0.7254 and 0.636, respectively.

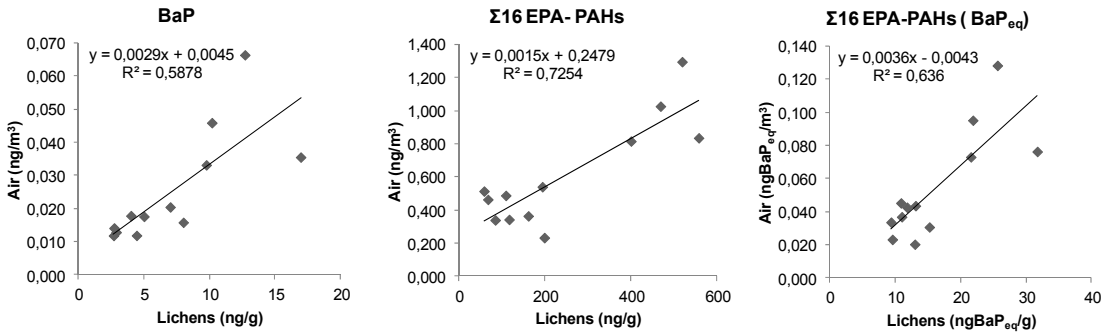

Figure 3. Relationship between lichens and the particulate-phase of air measured during 45 days prior to lichen sampling dates for: a) concentration of BaP; b) sum of the 16 EPA-PAHs; c) sum of the BaP equivalent concentrations.

DISCUSSION

PAHs in lichens in relation to air – a step towards calibration

For the first time it was possible to translate concentrations of PAHs in lichens into the equivalent ones for air, representing a step towards the fully integration of lichens into regulatory monitoring schemes.

The translation of BaP concentrations measured in lichens into the equivalent ones for the particulate-phase of air is important, as BaP is considered an indicator of carcinogenic risk and a surrogate of the concentration of the 16 EPA-PAHs (WHO, 2000). In terms of human health studies, the positive correlation between BaPeq concentrations (a measure of the toxic potency of the 16 PAH compound mixture) in

lichens and in particulate-phase of air opens new perspectives in the spatial assessment of human exposure to PAHs. Using the linear relationship built in this study it's possible to estimate human exposure through inhalation to toxic mixtures of PAHs with a high spatial resolution. Since lichens can be collected from a considerable number of sites, spatial models based on the translated BaPeq concentrations into the equivalents for air can be built and human exposure can be assessed with high detail in space. It will be possible to distinguish more accurately between control and exposed populations to this kind of pollutants. In this work we measured PAHs in TSP and the particles that are more detrimental for human respiratory systems are the smaller ones (< 2.5 μm). The small size particles have higher probability of reaching the deepest and most sensitive parts of human lungs compared to the larger ones (Vardar and Noll, 2003). Nevertheless, several studies have shown that TSP might be a proxy of particles of smaller sizes (Gomiscek et al., 2004). Thus, measuring PAHs in TSP, when other systems are not available, might give an idea of human exposure to PAHs present in total particulate matter and will help focusing health studies in specific spatial areas reducing the costs of looking to all the territory.

Besides the list of advantages of using lichens as biomonitors of atmospheric deposition already quoted in several works (Branquinho, 2001), another advantage of using lichens over the traditional monitoring, which is evidenced in this work, is the "memory" effect. Since lichens are reflecting a retroactive period of 45 days, it's possible to collect lichens after a PAH concentration peak has been detected using a conventional high-volume sampler, for example. If we collect the lichens in a spatial grid together with the interpretation of the PAH profile we are able to disclose, after the emissions, which sources were responsible for the high levels of PAHs in the atmosphere.

Recently European legislation fixed BaP maximum allowed concentration (measured in PM_{10} and averaged for one year) as 1 ng/m^3 (Directive 2004/107/EC) and this limit needs to be checked across the EU countries. Regarding BaP concentrations in particulate-phase of air (from 0.0040 to 0.1143 ng/m^3), our results are in the lowest range of estimated data previously published regarding PAH levels in Europe (Maliszewska-Kordybach, 1999). The highest concentrations of BaP in air were found in the former Czechoslovakia (2.9 ng/m^3), Hungary (1.9 ng/m^3), Germany (1.5 ng/m^3), Poland (1.4 ng/m^3) and Austria (1.3 ng/m^3); the lowest concentrations were found in

Finland (0.034 ng/m^3), Sweden (0.040 ng/m^3), Norway (0.042 ng/m^3) and Portugal (0.067 ng/m^3) (Maliszewska-Kordybach, 1999).

Concentrations of Σ16 EPA-PAH found in this study for lichens (varying from 58 ng PAH/g to 556 ng PAH/g) were in the range of those found by other authors that worked with lichens in countries such as Portugal, Spain and Italy (Augusto et al., 2009, 2010; Guidotti et al., 2003; Domeño et al., 2006; Blasco et al., 2006, 2007, 2008; Shukla and Upreti, 2009); whereas the ones measured in the particulate-phase of air (namely TSP) (0.077 ng PAH/m^3 and 1.156 ng PAH/m^3) were among the lowest values reported in other studies, where values have been estimated to be between 10-30 ng PAH/m^3 in central part of Europe and about 0.3-0.7 ng PAH/m^3 in the least polluted peripheral countries of the continent, such as Finland, Sweden, Norway and Portugal (Maliszewska-Kordybach, 1999). The lowest PAH values found for our study region may have several explanations. This region is a coastal area, in the most western part of Europe, with small cities with low population densities (the most populated city in the study region has 67 inhabitants/km^2) (INE, 2011). Moreover, industries present in the region have high chimneys (230 m) and are operating with the best available technologies for treating their atmospheric emissions; plus, direction of prevailing winds (from NW) brings clean airs from sea into inland and allows the quick dispersion and dilution of pollutants in the atmosphere.

Regarding total suspended particulate (TSP), we found the highest concentrations during summer months. This could be due to different reasons, namely i) during summer, meteorological conditions favor more pollutant and particle dispersions; this may be due to less rain events during summer, as rain washes out particles from atmosphere and promotes their deposition at shortest ranges (Smith and Harrison, 1998); ii) highest concentration of pollen during spring and summer months which are also intercepted by the filters and produce a confounding effect during this period. In this region the plants produce the highest levels of pollen during spring and summer.

On the contrary, a seasonal variation for both lichens and air, showing greater PAH concentrations (Σ16 EPA-PAHs) during winter and lower concentrations during summer was found in this work. Similar results have been reported in other studies and have been attributed to several reasons (Smith and Harrison, 1998; Panther et al., 1999). Some authors argue that there is an increase in emissions from domestic heating during winter (Smith and Harrison, 1998; Panther et al., 1999). Though this can be true for the

northern and central countries of Europe with very cold winters, in our region the energy production and distribution is quite constant over the year (INE, 2008). Other authors quote an increase of the levels of PAHs in winter due to traffic (from congestion and cold starts) (Smith and Harrison, 1998; Panther et al., 1999). However, cities in our region are small and with low population densities; moreover, this is a touristic region which becomes more densely populated during summer, thus traffic flow increases during summer, especially close to the most coastal areas.

Meteorological conditions, such as temperature, and photochemical reactions in summer, have been also used to explain the highest levels of PAHs during winter (Smith and Harrison, 1998; Panther et al., 1999). In fact, at higher temperatures and higher sunlight intensity which occurs during the summer we might have a stronger evaporation and degradation of PAHs (Beyer et al., 2003). Chemical and photochemical reactions with ozone or OH radicals are performed especially during the summer months (Schauer et al., 2003; Jung et al., 2010). In our particular study region, levels of ozone in the atmosphere have been reported to be relatively high ranging as an annual average between 60-80 μg/m3 (APA, 2012). This is mainly due to the presence of industrial and urban areas that produce NOx and VOCs and that, under high temperature and sunlight radiation, react in presence of oxygen producing ozone.

We found differences between the influence of temperature on concentrations of low molecular weight PAHs (LMW) and on high molecular PAHs (HMW) in the particulate-phase of air. It is commonly observed that HMW-PAHs are often associated with particulates while LMW-PAHs tend to be more concentrated in the vapour-phase (Zheng et al., 2000a,b). With high summer temperatures and high sunlight radiation the concentrations of PAHs in the vapour-phase increase, whereas during winter particulate-phase PAHs dominate (Bodzek et al., 1993; Masclet and Mouvier, 1988; Subramanyam et al., 1994; Wania and Mackay, 1996). In this study we didn't measure PAHs present in the vapour-phase of air, whose monitoring would involve the use of plugs of polyurethane foam (PUF) behind the filters to trap PAHs existent in the vapour-phase (the high-volume sampler that we used was not prepared to support this equipment). Nevertheless, during sampling using filters, PAHs (with vapour pressure less than 10–8 mm Hg, which correspond to the LMW-PAHs) may also be absorbed onto filter or onto particles on filters (phenomenon known as blow on) (Zhang and McMurry, 1991), however, as post-collection volatilisation may cause the loss of PAHs from the

filter (phenomenon known as blow off), it is essential to use the PUF system to efficiently retain these compounds. PAHs with five or more rings are almost exclusively absorbed on particulate matter collected on the filter, but the lower molecular weight PAHs are not fully retained, due to their volatility (Yamasaki et al., 1982). In this way, concentrations of LMW-PAHs in filters increase with increasing temperature, as there will be more LMW-PAHs associated to particles being captured by filters; nevertheless, vapour-phase concentrations of these compounds would be greatest if we had used the PUF system in our sampling.

Lichens showed significant negative correlations between temperature and all PAHs. Lichens are very efficient in intercepting pollutants from atmosphere; they're intercepting PAHs present not only in the particulate-phase of air, but also in the vapour-phase, as shown by their PAH profile. This characteristic was previously shown in other studies where PAH profile in lichens were compared to the ones for air, soil and pine needles in areas subjected to different kinds of pollution (Augusto et al., 2010). PAH profile in lichens is mostly dominated by 2-, 3-, and 4-ring PAHs, whereas PAH profile in particulate-phase of air is mainly dominated by 4-, 5- and 6-ring PAHs. Unlike filters, which were collected after 24h of exposure and kept protected from environmental factors (such as temperature, sunlight, etc.), lichens were continuously intercepting and accumulating PAHs. This means PAHs accumulated in lichens were subjected to all environmental factors until the day of their collection from the field; as lichens are intercepting both vapour- and particulate-phases of air, an increase of temperature may result in the volatilization of PAHs from lichens.

The obtained calibration between PAHs in lichens with PAHs in air should now be tested in other climates and with air particles from different sizes to evaluate whether we can have a more universal calibration or we need to adapt each calibration to specific regional conditions.

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Chapter 03 |

Fingerprinting pollution sources using biomonitoring tools

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3.1 | Spatial modelling of PAHs in lichens for fingerprinting of multi-source atmospheric pollution

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ABSTRACT

PAHs are toxic compounds emitted by several anthropogenic sources, which have a great impact on human health. We show, for the first time, how spatial models based on PAHs intercepted by lichens can be used for fingerprinting multi-source atmospheric pollution in a regional area. Urban-industrial areas showed the highest atmospheric deposition of PAHs followed by urban>industrial>agricultural>forest. Multivariate analysis of lichen data showed, for the first time, a clear distinction between various sources of PAHs in the same area: urban are dominated by 4-ring PAHs, forest by 3-ring PAHs and industrial by 5- and 6-ring PAHs or by 2-ring PAHs (petrogenic or pyrogenic, respectively). Heavy metals were also used for supporting the fingerprinting of PAH sources, reinforcing the industrial origin of 5- and 6-ring PAHs and revealing their particular nature. The spatial structure of the models for different PAHs seems to be dependent on the following factors: size and hydrophilic character of different PAHs, type of emission sources (point or non-point), and dispersion associated with particulates of different sizes. Based on the long-term integration of PAHs in lichens, these spatial models will significantly improve our knowledge on the impact of PAH chronic-exposure to humans and ecosystems.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic pollutants whose ubiquitous presence in ambient air is a recognized health concern (IARC, 1983; ATSDR, 1995). These compounds are highly lipophilic, and form mainly through incomplete combustion of fossil fuels and biomass, and spills of petroleum derivatives. Sources of PAHs in atmosphere include automobiles, re-suspended soils, refineries and power plants (Yang et al., 2002; Dyke et al., 2003). Due to the risk associated with human exposure to PAHs, especially through food and air, it is of crucial importance to develop strategies to identify sources of contamination in order to minimize the input of PAHs at ecosystem level and in the human food-chain; for that, not only must the sources be identified, but also the sites where PAHs are being deposited.

Chemical analyses of air, soil and plant and animal bio indicators have been used to monitor atmospheric deposition from different sources (Wolterbeek, 2002; Conti and Cecchetti, 2001; Nadal et al., 2004). While measurements in air (in the vapour- and particulate-phases) reflect a short-term indicator that varies considerably in space and

time, soils are sinks for organic compounds and therefore reflect a typical profile of long-term atmospheric pollution deposition. However, due to the volatilization of some compounds and the possible chemical changes of others, profiles of PAHs in soils should not be used to directly evaluate atmospheric deposition (Jonsson et al., 2007). The use of bio monitors, namely vegetation, offers a practicable, flexible and low-cost alternative against high costs associated with conventional monitoring using high volume air samplers. Different types of bio monitors have been used to monitor PAH concentrations in atmosphere, such as mosses, grasses and crops, herbs, garden vegetables and a variety of tree species (Srogi, 2007). Several authors have shown that PAHs in these biomonitors are more related to air deposition rather than to soil pollution (Augusto et al., 2010; Lehndorff and Schwark, 2004; Maliszewska-Kordybach, 2002; Migaszewski et al., 2002). Within biomonitors, lichens (symbiosis between fungi and algae and/or cyanobacteria) are one of the most used organisms to monitor atmospheric deposition of several air pollutants (Branquinho, 2001). Lichens are long-lived bio monitors and thus they are long-term integrators of the atmospheric pollution deposition. This characteristic is of crucial importance for evaluating human exposure to pollutants such as PAHs; time integration of these compounds allows relating low levels of pollutants with long-term chronic effects on health (Augusto et al., 2007). There are few studies showing that lichens can be used to monitor PAH atmospheric deposition (Augusto et al., 2010; Guidotti et al., 2003; Domeño et al., 2006; Blasco et al., 2006, 2007, 2008; Shukla and Upreti, 2009). Although some authors were not able to detect the most carcinogenic PAH compounds (5- and 6-ring PAHs) in lichen samples (Guidotti et al., 2003; Domeño et al., 2006), others have shown that lichens can be used to monitor PAH atmospheric deposition (Augusto et al., 2010; Blasco et al., 2006, 2007, 2008; Shukla and Upreti, 2009). Blasco et al. (2008) used lichens and found that the road traffic was the main source of PAHs in the Pyrenees Mountains region. These authors found that PAHs in lichens reflected the atmospheric particulates when they studied the PAH pollution caused by vehicle emissions in a tunnel (Blasco et al., 2006). More recently, when comparing lichens to soil and air, it was shown that PAH lichens' profile was substantially different from that of the soil, but similar to that of the air; it was also revealed that lichens intercept PAHs both from the vapour- and particulate-phases of air (Augusto et al., 2010). However, to our knowledge, no study has been performed using spatial mapping of PAHs in lichens for fingerprinting multiple sources of atmospheric PAHs in a regional area. The majority of the studies were done collecting lichens in a

well-known restricted area, linked to a specific type of pollution. However, they did not show how lichens could be used to distinguish between different pollutant sources in a multiple source environment.

Multivariate analyses (such as factor analysis and principal component analysis) are frequently used for source apportionment. These tools are used to reduce a set of numerous original variables to two or three variables, which capture the main patterns by eliminating redundant information in data; they have been used as an exploratory tool to identify the major sources of PAH emissions and select statistically independent source tracers (Simcik et al., 1999). The contribution of each of the sources could be distinguished by their different physical and chemical properties.

The methods described above for source apportionment can determine the types and strength of pollution sources, but do not characterize the spatial patterns of PAHs. Geostatistics provide an appropriate framework to characterize the spatial distribution of variables and the associated uncertainty. The spatial variability of variables is characterized through the inference and modelling of the semivariogram, which is afterwards used in the estimation and simulation routines for mapping. The simple interpretation of the semivariogram model provides insight on the spatial continuity (Isaaks and Srivastava, 1989). Moreover, the existence of a model of spatial dependence allows the estimation of attribute values at unsampled locations through the use of kriging algorithms (Goovaerts, 1997). Therefore, geostatistics can be used to build maps of attributes, such as PAH concentrations or PAH concentrations ratios, which are valuable tools for decision makers and managers. These methodologies have been widely used for modelling atmospheric pollutants, including those based on lichen data (Augusto et al., 2004; Pinho et al., 2008a,b).

Although this issue has been discussed in a number of publications regarding PAHs and their different sources (mainly urban and/or industrial), the relationships between PAH profiles and quantitative variables based on land-use have not yet been examined. The majority of studies dealing with PAHs in soil, air or vegetation, covers areas where there is a dominant land-use class, namely urban, industrial or natural areas; however, these studies have not encompassed regional areas with multiple land-use classes and multi-sources of atmospheric pollution. As a consequence, most data characterize a type of source without being able to directly distinguish the different land-uses classes in the same study.

The main aims of this study were: i) to establish relations between PAH profile in lichens and different land-uses, in order to identify specific profiles in lichens as surrogates of specific sources in a multi-pollutant environment; ii) to develop geostatistical spatial models for PAH deposition using lichens as bio monitors, in order to obtain high resolution maps and test the best indicators based on their spatial continuity. The results obtained in this study will provide know-how regarding the evaluation of human and ecosystem long-term exposure to the PAH toxic compounds.

EXPERIMENTAL SECTION

Description of the study area

This study was developed in the highly industrialized region of Sines, located on the SW coast of continental Portugal, facing the Atlantic Ocean. This region encompasses several important industrial facilities established in the late 1970's: a coal-fired power station, an oil refinery, a chemical plant, and more recently an industrial landfill, as well as many other smaller industrial plants, based primarily on the processing of oil products. Moreover, an urban area has recently increased (Figure 1). Three main cities can be found in the area, namely Sines (with an area of 151 km^2 and 12 461 inhabitants), Santiago do Cacém (with 120 km^2 and 7 274 inhabitants) and Santo André (with 75 km^2 of area and 10 696 inhabitants).

Sampling

In January 2008 (during 3 days under constant climatic conditions, with an average temperature of 14 ºC, an average precipitation of 0.1 cm and an average humidity of 96 %), 34 lichen samples of the species *Parmotrema hypoleucinum* (Steiner) Hale were collected at a number of sites within the highly industrialized region of Sines. The sampling design followed a two kilometre grid that had previously been selected for different studies (Pinho et al. 2008b). The lichen *P. hypoleucinum* was selected because it is ubiquitous and tolerates a variety of land-uses, such as urban, industrial, forestry and also background areas. The collection was made mainly from branches and trunks of *Quercus suber* L. (cork-oak) (N=25), from *Pinus pinea* L. (umbrella-pine) (N=4) and some exceptions from other tree species (especially in urban areas). Samples were packed in brown glass bottles, protected from sunlight and immediately stored at 4ºC. All samples were extracted and analysed for the 16 EPA-PAHs within two months.

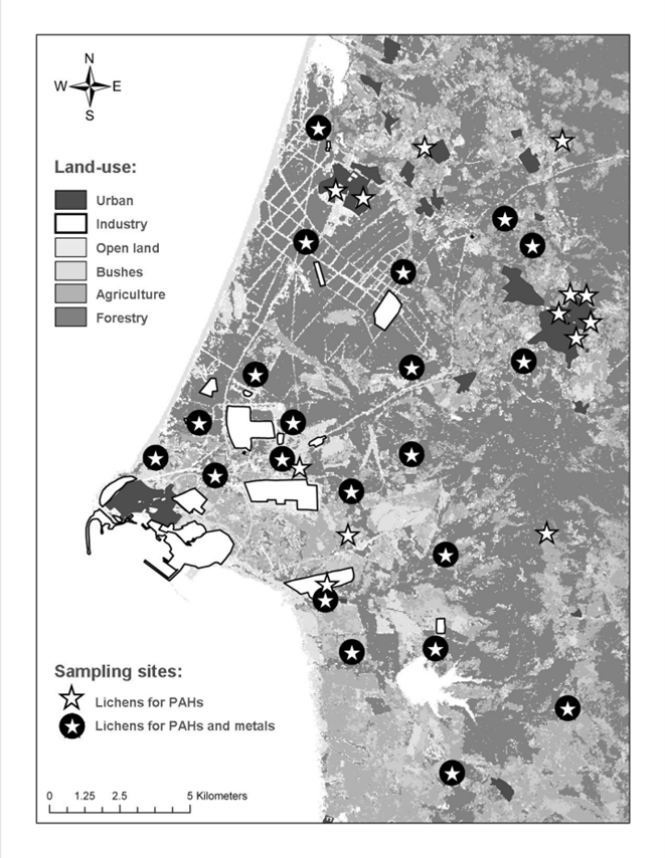

Figure 1. Map of the study area – Sines, in the southern part of Portugal, Europe – showing the land-use classes and the location of the lichen sampling sites for PAHs analysis (N=34) and for metal analysis (N=21). Land-use classification was made by assisted classification of LandSat images (30 m resolution, multispectral, acquired in spring of 2001) (Pinho et al., 2008a,b).

Sample preparation and analysis

All PAH analyses took place at the certified laboratory of the Portuguese Environmental Protection Agency (APA). Approximately 2 g of sample was placed in a Soxhlet with 200 mL of acetonitrile (HPLC grade) for 24 h. After the extraction of the PAHs from the samples, the extracts were concentrated by rotary vacuum evaporation and cleaned-up in a florisil column with 30 mL of acetonitrile as eluting solvent. Subsequently, the extracts were again evaporated and concentrated with a gentle stream of purified N_2 to 1 mL. The samples were analysed by a high-performance liquid chromatograph (Hewlett Packard), using two columns (Agilent C18 and Phenomenex C18), coupled to an ultraviolet fluorescence detector (FLD) and to an ultraviolet/visible detector (DAD/V-

UV). The acetonitrile/water gradient profile was 50:50 for 5 min, 60:40 over 15 min, 90:10 for 4 min, 80:20 during 6 min, 90:10 for 10 min, and finally 100:0 over 5 min at a flow rate of 1 ml/min. Column temperature was kept at 28 ºC.

The sixteen EPA-PAHs were analyzed, namely: acenaphtylene, naphthalene, fluorene, phenanthrene, fluoranthene, chrysene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, acenaphtene, anthracene, pyrene, benzo[a]pyrene and indeno[1,2,3-cd]pyrene. The majority of compounds presented concentrations above detection limit, except acenaphtylene which presented values under detection limit. PAH standards of Ultra-scientific with an uncertainty of 5 % were used.

Metal concentrations in lichens from previous studies

In September 2002, lichens of the species P*. hypoleucinum* were collected at 21 of the 34 sampling sites where lichens were collected for PAH analyses (Figure 1), mainly from trunks and branches of *Pinus pinea* L. and *Quercus suber* L. After collection, the lichens were stored in plastic bags and transported to the laboratory, where the unwashed samples were immediately dried at room temperature and sorted to remove extraneous material. Special care was taken when sorting in order to select only the chosen species, to avoid errors due to the presence of other species. The cleaned samples were then ground (Glen Creston Ltd. MM, 2000) and separated into two parts: one for metal analysis (Si, Ni, Mn, Ca, Cr, Al, K, Fe, Co, Ti, Zn and Mg) and another for S and N. For S and N analysis, ground lichen samples were dried at 50 °C for 24 h. Three replicates of each sample were separated (2.5 mg for S analysis and 1 mg for N analysis) in a high-performance balance (Sartorius Microanalytical Balance) and analyzed by elemental mass analysis (Euro Vector CHNS-O Elemental Analyzer). The standards used were atropine ($C_{17}H_{23}NO_3$ with 4.84% N) for nitrogen analysis and BBOT ($C_{26}H_{26}N_2O_2S$ with 7.44% S) for sulphur analysis. The precision of analysis was 0.03% for S and 0.07% for N, and the accuracy was 0.02% for S and 0.01% for N. For metal analysis, ground and dried samples were submitted to an extraction by boiling with concentrated aqua-regia (hydrochloric and nitric acids) for up to 3 hours, and quantified by ICP-OES. The precision and accuracy of the analysis was checked against reference material. These metal analyses took place in the British analytical laboratory Tes Bretby. All metal analyses performed are covered by our UKAS Accreditation.

Statistical analysis

Statistical analysis of the results was carried out using the statistical STATISTICA 8.0 StatSoft Inc. package. For samples in which a compound was not detected (ND), its concentration was assumed to be zero. A principal component analysis (PCA) was performed for PAH profiles (contribution of each compound, or each group of rings, for the sum of the 16 EPA-PAH) and the area covered by each class of the land-use at each sampling site within buffers of increasing radii (from 500 to 10000 m). The land-use classes considered were: urban, industrial, agricultural, open land, forest and bush areas. For spatial visualization, the obtained site scores of the PCA were modelled as described in the next section. Pearsons' linear correlations between PAH profiles and metal concentrations in lichens were also calculated. A 95% level of significance was considered for the results of all correlations.

Spatial modelling

In a first step, spatial correlations between samples were generalized in a correlation function of distance between any two points, the semivariogram, which summarizes the main spatial continuity patterns of the attributes. For this purpose we used spherical and exponential models, which belong to a set of simple "basic models" that are often referred to as transition models; these types of models reach a plateau that is called *sill*, *C*, at a distance called *range, a*. The spherical and exponential models are defined in their isotropic form by eq. 1 and eq. 2:

$$\gamma_{sph}(h) = \begin{cases} C\left[1.5\frac{h}{a} - 0.5\left(\frac{h}{a}\right)^3\right] & if\ \ h \le a \\ 1 \quad otherwise \end{cases} \qquad (1)$$

$$\gamma_{\exp}(h) = C\left[1 - \exp\left(\frac{-3h}{a}\right)\right] \qquad (2)$$

The range is the most important parameter because it is related to the spatial extent of continuity of the phenomenon (Isaaks and Srivastava, 1989; Goovaerts, 1997).

In a second step, a least-squares linear regression algorithm, the ordinary kriging, was applied to estimate grid maps for each attribute taking into account the models spatial dependence previously fitted. Let $\{z(\mathbf{u}_\alpha), \alpha = 1, \dots, n\}$ be the set of n measurements of

the continuous attribute z located at $\mathbf{u}_\alpha$ over study area A. The ordinary kriging estimator $Z^*(\mathbf{u})$ is a linear combination of the $n(\mathbf{u})$ random variables $Z(\mathbf{u_\alpha})$:

$$Z^*(\mathbf{u}) = \sum_{\alpha=1}^{n(\mathbf{u})} \lambda_\alpha(\mathbf{u}) Z(\mathbf{u_\alpha}) \quad \text{with} \quad \sum_{\alpha=1}^{n(\mathbf{u})} \lambda_\alpha(\mathbf{u}) = 1$$

where $\lambda_\alpha(\mathbf{u})$ is the weight assigned to datum $z(\mathbf{u_\alpha})$ interpreted as a realization of the random variable $Z(\mathbf{u_\alpha})$ under the constraint of unbiasedness of the estimator

$$E\{Z^*(\mathbf{u}) - Z(\mathbf{u})\} = 0$$

The $n(\mathbf{u})$ weights $\lambda_\alpha(\mathbf{u})$ are determined by minimizing the error variance, $\sigma_E^2(\mathbf{u})$,

$$\sigma_E^2(\mathbf{u}) = Var\{Z^*(\mathbf{u}) - Z(\mathbf{u})\} = E\left\{\left[\sum_{\alpha=1}^{n(\mathbf{u})} \lambda_\alpha(\mathbf{u}) Z(\mathbf{u_\alpha}) - Z(\mathbf{u_\alpha})\right]^2\right\}$$

Once the semivariogram has been defined and accounting for the relation $C(\mathbf{h}) = C(0) - \gamma(\mathbf{h})$, where $C(\mathbf{h})$ is the spatial covariance model, $\sigma_E^2(\mathbf{u})$ can be written as:

$$\sigma_E^2(\mathbf{u}) = C(0) + \sum_{\alpha=1}^{n(\mathbf{u})} \sum_{\beta=1}^{n(\mathbf{u})} \lambda_\alpha(\mathbf{u}) \lambda_\beta(\mathbf{u})\, C\left(\mathbf{u_\alpha}, \mathbf{u_\beta}\right) - 2 \sum_{\alpha=1}^{n(\mathbf{u})} \lambda_\alpha(\mathbf{u}) C(\mathbf{u_\alpha}, \mathbf{u})$$

Hence, using the matrix notation, the solution of the ordinary kriging system is written as

$$[\lambda] = [K]^{-1} \cdot [M]$$

where $[\lambda]$ is the vector of kriging weights, $[K]$ is the matrix of data covariances and $[M]$ is the vector of data-to-unknown covariances.

RESULTS AND DISCUSSION

PAH levels in lichens

In this work we found that concentrations of the 16 EPA-PAHs were in the range of those found by other authors that worked with lichens (Augusto et al., 2010; Guidotti et al., 2003; Domeño et al., 2006; Blasco et al., 2006, 2007, 2008; Shukla and Upreti, 2009) (Table 1).

TABLE 1. PAH concentrations (ng PAH/g lichen) measured in lichens collected from sites with different dominating land-use classes within a buffer of 1 km centred at each sampling site: industrial sites (0.93 to 18.98% covered by industrial areas, N=9), urban sites (0.11 to 43.87% covered by urban areas, N=8), industrial and urban mixed sites (21.40 to 64.64% covered by industrial and urban areas, N=6), forest sites (50.45 to 71.91% covered by wooded areas, N=6) and agricultural sites (27.78 to 47.48 % covered by agricultural areas, N=5).

		industrial	urban	ind + urb	forest	agriculture
2-ring PAHs	average	20.9	20.1	64.0	16.4	25.1
	SD	14.5	3.8	70.2	5.6	8.0
	min	18.3	13.6	14.9	10.6	13.5
	max	58.8	25.9	175.3	25.6	33.3
3-ring PAHs	average	56.3	74.4	112.5	38.5	55.8
	SD	22.1	40.0	59.5	10.9	20.8
	min	32.3	37.5	65.2	27.7	38.9
	max	91.3	137.1	217.9	54.8	88.4
4-ring PAHs	average	75.1	180.4	226.2	58.8	91.9
	SD	35.0	133.2	77.1	20.0	21.6
	min	32.4	60.6	127.9	27.7	68.5
	max	149.0	426.2	325.9	85.3	124.4
5-ring PAHs	average	11.5	11.2	33.6	7.7	13.5
	SD	10.0	3.0	30.7	4.9	6.0
	min	2.8	6.8	11.0	4.3	5.4
	max	35.7	16.4	86.6	17.4	21.7
6-ring PAHs	average	6.9	3.7	18.3	2.1	4.6
	SD	7.6	1.3	24.9	0.8	3.8
	min	0.9	1.6	3.1	1.1	0.0
	max	24.0	4.8	66.2	3.4	8.9
16 EPA-PAHs	average	178.9	289.7	454.5	123.3	191.0
	SD	80.0	174.5	233.9	30.2	50.7
	min	93.6	126.8	232.9	90.5	128.4
	max	332.0	599.2	871.8	157.1	264.9

[a] Industrial sites (0.93 to 18.98% covered by industrial areas, $N = 9$), urban sites (0.11 to 43.87% covered by urban areas, $N = 8$), industrial and urban mixed sites (21.40 to 64.64% covered by industrial and urban areas, $N = 6$), forest sites (50.45 to 71.91% covered by wooded areas, $N = 6$), and agricultural sites (27.78 to 47.48% covered by agricultural areas, $N = 5$).

Lichens collected from single urban areas showed greater concentrations for the 16 EPA-PAHs, varying between 126.78 and 599.24 ng PAH/g lichen, when compared to those collected from single industrial areas, where values ranged from 93.56 to 332.00 ng/g (Table 1). The highest concentrations were found for mixed site industrial-urban areas, where values ranged from 232.87 to 871.83 ng/g. These mixed areas also presented higher concentrations for each group of PAH rings, compared to other land-use classes (Table 1). In contrast, lichens from background areas (primarily forests) showed the lowest concentrations for the 16 EPA-PAHs, ranging between 90.49 and 157.12 ng/g, followed by agriculture areas with values ranging from 128.41 to 264.90 ng/g (Table 1). These results showed that urban and industrial areas seem to be

responsible for the major input of PAHs in the environment, as the highest PAH concentrations were found for lichen samples collected at these areas. Moreover, PAHs from industrial areas seem to be added to the ones of urban areas, increasing the overall concentration of the 16 EPA-PAHs at these mixed areas (Table 1).

Multisource fingerprinting

One of the aims of this study was to establish relationships between PAH profiles in lichens and the land-use, in order to be able to identify specific profiles as surrogates of specific pollution sources, such as urban and industrial. The most widely used method in literature for appointing sources is the gradient method which is based on the assumption that pollutant levels increase with increasing proximity to the suspected source (Becket et al., 1982). However, in a multiple source environment, where urban areas are mixed with industrial areas, these commonly measurements (e.g. distance to a specific source) should not be used to relate pollution sources to a certain sampling site.

Lichens like any other organisms are influenced by multiple sources of pollution. If a sampling site is located in a protected area, such as a dense forest, it will be less affected by pollution compared to that located in an exposed area with uncovered soil. Lichens collected from these different sampling sites will present different pollutant profiles and concentrations, although both sampling sites can be located at the same distance from a known pollution source. In this way, it is crucial to evaluate the contribution of land-use to the distribution pattern of pollutants in lichens. For that purpose, we have calculated the relative cover of each land-use class in circular buffers with several radii centred at each sampling site. The land-use classes present at the study area are: industrial, urban, forest, open land (areas without or with little vegetation), bushes and agricultural. In order to find relationships between the land use class and PAHs concentrations in lichens, we performed principal component analyses (PCA). We propose to use the relative concentrations of PAH rings or compounds (percentage to the sum of the concentration of the 16 EPA-PAHs), rather than concentrations, as they are more indicative of pollutant sources; concentrations per se only give information on the gradient of pollution without indicating any changes in the proportion of each PAH compound, or any pollution sources. We have used buffer radii from 500 m to 10000 m for evaluating the impact of buffers size, and results show that buffers between 500 and 2000 m are similar and consistent (data not shown). Here, we only show the results for buffers with 1000 m radius.

Regarding the PAH ring profiles, the first two principal components extracted, PC1 and PC2, accounting for 58.24 % of the variance, are displayed in Figure 2A. PC1 is mostly positively weighted by 4-ring PAHs and by urban areas and negatively weighted by industrial and open land areas and also by 2-, 5- and 6-ring PAHs. PC2 is positively weighted by 3-ring PAHs and forestry areas and negatively weighted by industrial and open land areas and by 5- and 6-ring PAHs (Figure 2A). These results clearly show a distinction between PAH profiles of urban, industrial and forestry areas; whereas PAH profile is dominated by 4-ring PAHs in urban areas, in forestry areas the profile is dominated by 3-ring PAHs and in industrial areas it is undoubtedly dominated by 5- and 6-ring PAHs. The 2-ring PAHs have not a clear association with any land-use class because, probably the land-use classes have sufficient discrimination to capture the pattern or because they can be related to a particular industry type. Its association to PC1 reveals that there is an association with industry, but its separation by PC2 discloses that there are different patterns between industries (Figure 2A). The site scores associated with the 2-ring PAHs profile (namely naphthalene) are in fact located next to a refinery and a coal power plant, which can be related to both petrogenic and pyrogenic emissions (Rao et al., 2008). The association between open land areas and areas with herbs or bushes and the 5- and 6-ring PAHs might be related to wind-blown dust in these exposed areas together with some soil ressuspension and also as a consequence of volatilization of lighter aromatic hydrocarbons as a result of greater exposition to solar radiation and a higher temperature (Korcz et al., 2009; Laidlaw and Filippelli, 2008).

The first two components (PC1 and PC2) of the PCA performed for the PAH compound profiles, accounting for 49.87 % of the variance, are displayed in Figure 2B. It is interesting to note that the 4-ring PAH compounds associated with urban areas are mainly fluoranthene, pyrene and chrysene, although the 3-ring PAH anthracene appear to be associated with urban areas (see PC1 in Figure 2B). Industrial and open land areas showed association with the 4-ring PAH benzo[a]anthracene and with all the 5- and 6-ring PAH compounds, except with the 5-ring PAH dibenzo[a,h]anthracene (see PC1 in Figure 2B). The forestry areas appeared highly associated to the 3-ring PAH phenanthrene, a compound that has been identified as typical of background pattern in soils (see PC2 in Figure 2B) (Wilcke, 2007).

The study area includes a variety of industries, notably a coal-fired power station, an oil refinery, a chemical plant, an industrial landfill and other smaller industrial plants and facilities primarily based on the processing of oil products. In addition, some urban areas are located close to these industrial areas, making it even more difficult to identify what is emitted by industries or by urban activities. Nevertheless, our results clearly show that there is an association between the PAH profiles measured in lichens and the each land-use. It is possible to distinguish industrial profiles, characterized by 2- , 5- and 6- ring PAHs (especially naphthalene, benzo[k]fluoranthene, benzo[g,h,i]perylene and indeno[1,2,3-cd]pyrene), from urban profile, characterized by 4-ring PAHs, such as fluoranthene, pyrene and chrysene. These 4-ring PAH compounds, in addition to anthracene, are known to be present in vehicle emissions (Guidotti et al., 2003). In this study the 2- and 3-ring PAHs (low molecular weight PAHs) were separated from the 5- and 6-ring PAHs in the PCA (Figure 1A). The 2-ring PAHs are usually emitted from petroleum and its products without combustion, and from purification of oil products, whereas the high molecular PAHs (especially 5- and 6-ring PAHs) often come from coal and fuel combustion and coke manufacturing (Bixiong and Zhang, 2007). Our results clearly show that with PAH profiles in lichens it is possible to distinguish, not only urban from industrial sources, but also different industries (petrogenic and pyrogenic) (Figure 2).

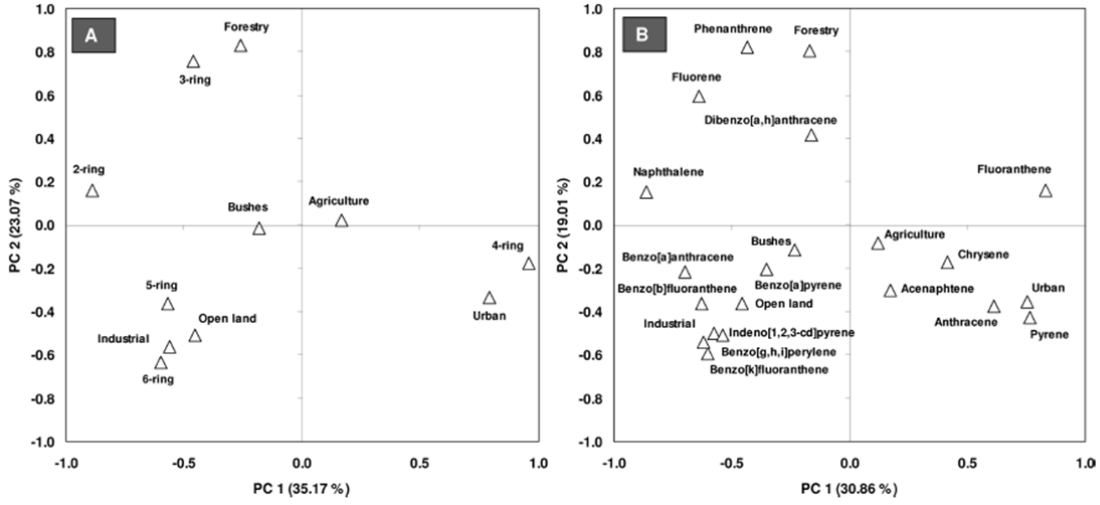

Figure 2. Principal component analysis (PCA) of the area occupied, within a 1 km buffer centred in each sampling site, by the following classes of the land-use: urban, industrial, agricultural, open land, forest and bush areas; and A) the PAH ring profile measured in

lichens collected at 34 sampling sites; B) the PAH compound profile measured in lichens collected at 34 sampling sites in the study. The compound acenaphtylene was not considered in the analysis because it was below detection limit.

In this work it was not possible to make any comparison with previously published lichen data, because the authors didn't analyze the PAH profile for different emission sources (Augusto et al., 2010; Guidotti et al., 2003; Domeño et al., 2006; Blasco et al., 2006, 2007, 2008; Shukla and Upreti, 2009). However, the relationships between each PAH compound and its sources (here land-use) are generally similar to those obtained from other environmental systems, e.g. air, soil and vegetation (Srogi, 2007).

Heavy metals as surrogates for atmospheric particulate deposition

Different chemical compounds can be used in order to confirm the origin of atmospherically deposited pollutants. In this work, we used the concentration of heavy metals to help in the process of fingerprinting of PAH sources. Moreover, PAHs are compounds that might be in the air-phase and/or as particulates. In general low molecular weight compounds (2, 3 and some 4 rings) might have a higher proportion in the air-phase than the high molecular ones (5, 6 and some of the 4 rings). However, during deposition process PAH compounds might be bound to larger particulates. This is important since large particulates might have a different pattern of deposition and a different impact on ecosystems and human health; as for example a different bioavailability for the biological systems. Moreover, PAHs have often been found to be associated with heavy metals due to similar pollution sources (Mielke et al., 2004). Pearsons' linear correlations between PAH profiles in lichens from this study and metal concentrations in lichens obtained from the same region but in previous studies were performed and results are presented in Table 2.

Although with moderate correlations, it can be observed that 5-ring PAHs are significantly positively correlated with Cr, Al and Fe; 6-ring PAHs are significantly positively correlated with Cr and Zn; and 3-ring PAHs are negatively correlated with Pb and Zn. Considering PAH individual compounds, Cr showed to be positively correlated with benzo[a]pyrene and indeno[1,2,3-cd]pyrene; Zn showed positive correlations with benzo[k]fluoranthene, benzo[g,h,i]perylene and indeno[1,2,3-cd]pyrene and negative correlations with phenanthrene and dibenzo[a,h[anthracene; Al and Fe presented positive correlations with benzo[b]fluoranthene and benzo[a]pyrene.

TABLE 2. Significant Pearson R for correlations between PAH profiles and heavy metal concentrations measured in lichens. N= 21.

	Cr	Pb	Zn	Al	Fe
2-ring PAHs	n.s.	n.s.	n.s.	n.s.	n.s.
3-ring PAHs	n.s.	**−0.55**	**−0.44**	n.s.	n.s.
4-ring PAHs	n.s.	n.s.	n.s.	n.s.	n.s.
5-ring PAHs	**0.45**	n.s.	n.s.	**0.58**	**0.55**
6-ring PAHs	**0.48**	n.s.	**0.61**	n.s.	n.s.
naphthalene	n.s.	n.s.	n.s.	n.s.	n.s.
fluorene	n.s.	n.s.	n.s.	n.s.	n.s.
phenanthrene	n.s.	**−0.56**	**−0.49**	−0.33	n.s.
fluoranthene	n.s.	n.s.	n.s.	n.s.	n.s.
chrysene	n.s.	n.s.	n.s.	n.s.	n.s.
benzo[a]pyrene	n.s.	n.s.	n.s.	n.s.	n.s.
benzo[b]fluoranthene	n.s.	n.s.	n.s.	**0.50**	**0.44**
benzo[k]fluoranthene	n.s.	n.s.	**0.53**	n.s.	n.s.
dibenzo[a,h]anthracene	n.s.	n.s.	**−0.44**	n.s.	n.s.
benzo[g,h,i]perylene	n.s.	n.s.	**0.54**	n.s.	n.s.
acenaphtene	n.s.	n.s.	n.s.	n.s.	n.s.
anthracene	n.s.	n.s.	n.s.	n.s.	n.s.
pyrene	n.s.	n.s.	n.s.	n.s.	n.s.
benzo[a]pyrene	**0.45**	n.s.	n.s.	**0.55**	**0.51**
indeno[1,2,3-cd]pyrene	**0.53**	n.s.	**0.55**	n.s.	n.s.

[a] $N = 21$. n.s. = no significant. Bold: significant $p < 0.05$.

Aluminum and Fe are considered indicators of particulate matter and thus 5-ring PAHs might be deposited with particulate matter in lichens, as they correlate well with these metals. The correlation of 5- and 6-ring PAHs with Cr and of 6-ring PAHs with Zn might indicate an industrial influence, as confirmed by the association of these PAHs with industrial areas (Table 2, Figure 2). The release of metals such as Cr may occur in refining operations and burning of residual oils (Nadal et al., 2009). Also, Zn in addition to Cr, are characteristic of coal combustion in power generating plants (Huang et al., 2004) located in the study area.

The negative correlation between 3-ring PAHs (especially phenanthrene) and Pb and Zn, in addition to their association with forestry areas (Figures 2) and low values for the sum of the 16 EPA-PAH concentrations (Table 1), might indicate that these PAHs are related to areas without anthropogenic pollution or background areas (Table 2 and Figure 2). This is in accordance to other authors that claimed that phenanthrene has been identified as typical PAH marker of background pattern (Wilcke, 2007).

Spatial modelling of PAHs fingerprints

The second aim of this work was to develop geostatistical models for PAH deposition using lichens as biomonitors, in order to obtain high resolution maps and test the best compound indicators based on their spatial continuity. Considering the emission

sources show diverse PAH distribution patterns, investigation of the spatial variability of PAH profiles allows elucidation of their origin.

For this purpose, we first modelled the spatial continuity, using variography analysis, of the PAH ring and compound profiles and the site scores (PC1 and PC2) obtained from the PCA made in the previous section for the ring profile. First experimental semivariograms were calculated from data values and then a model was fitted to each one (Table 3). The main objective is to capture in the semivariogram model the spatial pattern of the physical phenomenon rather than getting a best fit of a second moment. Thus, all models were modelled by a visual fitting procedure using a graphical interface. Some of the variables were fitted by spherical models and others by exponential models; the exponential model shows a rapid decrease of continuity close to the origin (short distances) when compared to the spherical model. In this case, due to the relative small number of samples in space it was not possible to identify anisotropies. The parameters of the semivariogram model (sill and range) are used to assign optimal weights for spatial prediction using kriging, i.e., to interpolate and map the variables for the entire study area. Results are presented in Table 3 and Figure 3A,B.

The results show that 5- and 6-ring PAHs present lower spatial continuities (given by their range), namely 5000 and 2500 m, respectively, when compared to 2- and 4-ring PAHs, which display continuities of 10000 m and 9000 m respectively (Table 3).

As discussed in the previous section, 5- and 6- ring PAHs might be of industrial origin and associated to particulates and thereby tend to be deposited closer to emission sources. On the other hand, 2-ring PAHs can also be emitted by industries, but as compounds of low molecular weight, they tend to be ubiquitous pollutants with a long-range transport capacity (Nadal et al., 2009; Meharg et al., 1998), as shown by long range spatial continuity found in this study (Table 3).

Four ring PAHs that occur in urban areas, showed a spatial continuity higher than 5- and 6-ring PAHs, most probably because they are emitted by a set of diffuse and mobile sources usually present in urban areas. This high range of the 4-ring PAHs is due to fluoranthene which presents a range of 12 500 m, much greater than the other 4-ring compounds, such as chrysene and pyrene, which showed values less than 5000 m (Table3). The great difference found in the range of the spatial continuity of the compounds emitted by urban areas might be related to the association to different

particulates during the deposition process. The ambient aerosol characteristic from urban sites shows a bimodal distribution of particulates: the coarse fraction (crustal material, paved-road dust, non-catalyst equipped gasoline engines) and the fine fraction (mixture of primary and secondary aerosol emitted from anthropogenic rather than natural sources or formed by vapour nucleation/condensation mechanisms) (Aceves and Grimalt, 1993; Kleeman and Cass, 1998; Hildemann et al., 1991, as suggested by our observations (Table 3).

TABLE 3. Semivariogram models for PAH ring and compound profiles measured in lichens and log octanol-water partitioning coefficients for each PAH compound (K_{ow}) (Meharg et al., 1998). * Range not calculated, since the compound presented small values of variance.

	K_{ow}	range (m)	function
naphthalene	3.29	8500	exponential
fluorene	4.18	3000	spherical
phenanthrene	4.45	3500	spherical
anthracene	4.45	1000	spherical
acenaphtene	9.98	1000	spherical
pyrene	4.88	5000	exponential
fluoranthene	4.90	12500	spherical
chrysene	5.16	3000	spherical
benzo[a]anthracene	5.61	8000	exponential
benzo[b]fluoranthene	6.04	9000	exponential
benzo[a]pyrene	6.06	3700	spherical
benzo[k]fluoranthene	6.06	2500	exponential
dibenzo[a,h]anthracene	6.84	*a*	*a*
benzo[g,h,i]perylene	6.50	4000	spherical
indeno[1,2,3-cd]pyrene	6.58	1500	spherical
2-ring PAHs		10000	exponential
3-ring PAHs		4000	exponential
4-ring PAHs		9000	spherical
5-ring PAHs		5000	spherical
6-ring PAHs		2500	spherical
PC1 (PAH rings)		12000	spherical
PC2 (PAH rings)		5000	spherical

[a] Range not calculated, since the compound presented small values of variance.

Considering the low molecular weight PAHs, 2- and 3-ring PAHs (the least hydrophobic compounds), it should be noted that the range of the spatial continuity follows the tendency of the octanol-water partitioning coefficients (K_{ow}); the spatial range of these compounds decrease with the increase of the hydrophobicity. On the other hand, the high molecular weight PAHs (the most hydrophobic compounds) are most dependent on the type of particulate to which they are adhered and on the type of emission source (point or non-point).

Semivariogram models of the PCA scores (for the PAH ring profile), which represent a summary of the previous data, show that PC1 presented larger spatial continuity than the majority of the single PAH rings and compounds (Table 3), whereas PC2 showed a small spatial continuity. In the interpolated map of the PC1 (Figure 3A) it is possible to distinguish the urban (darkest areas) from the industrial areas (greyest areas) and locate them in the study region. The darkest areas correspond to the main cities present in the region, whereas the greyest ones correspond to the main industrial areas. In the map of PC2 it is possible to observe that darkest areas correspond to the main forestry areas present in the region (Figure 3B).

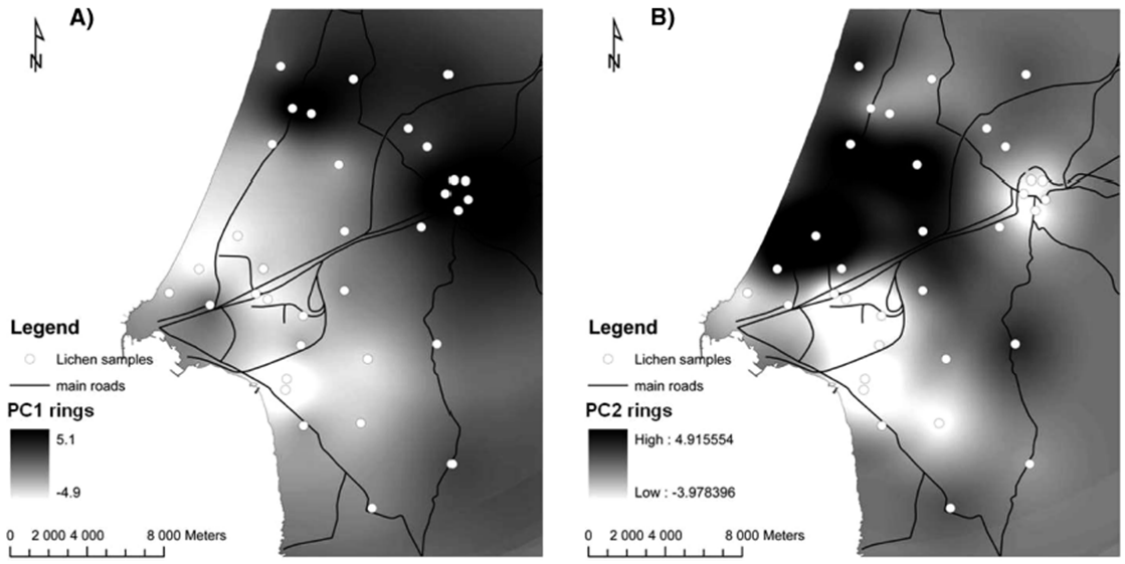

Figure 3. A) and B) Map of the interpolation of the site scores (PC1 and PC2, respectively) obtained through the PCA made with PAH ring profiles measured in lichens and the land-use.

Our study show for the first time how spatial models based on PAHs intercepted by lichens can be used for fingerprinting multi-source atmospheric pollution in a regional area. This study could work as a basis for the use of lichens as tracers of PAHs origin, namely of urban and industrial areas in other parts of the world. The knowledge of the extent of atmospheric deposition of specific PAHs from specific sources, obtained in this model, could be applied in environment impact studies. The same information could also be used for validating air deposition models for PAHs, since it is based in high spatial

resolution information. At the local level this work could be useful for evaluating the impact of PAHs from different sources in different ecosystem compartments. Based on the model it is possible now to select areas for evaluating the impact of PAHs atmospheric deposition on: soil, groundwater and animal food-chain contamination. This work will also be useful for local territory management since this is an industrial area (*e.g*. helping in the location of a new industry). These spatial models would be useful in environmental-health studies for calculation the human chronic-exposure to environmental PAHs and evaluate the contamination of human food-chain.

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3.2 | Evaluating sources of PAHs in urban streams based on land-use and biomonitors

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ABSTRACT

Toxic polycyclic aromatic hydrocarbons (PAHs) can be found in wastewaters and sewages released from industries and/or urban areas. When discharged untreated to stream waters, they can be a problem to human health. This work represents the first attempt to use PAH and metal concentrations in aquatic moss transplants together with land-use information to identify water pollution sources in urban areas. To do this, the moss *Fontinalis antipyretica*, was collected from a natural stream and transplanted to four different streams in a densely populated area of Lisbon, Portugal. After three months of exposure, mosses were collected and analyzed for metals and for the 16 priority PAHs recommended by the US EPA. Urban streams seem have a scattered contamination of 6-ring PAHs. Correlations between land-use, metal concentrations, and PAH concentrations indicated that areas occupied by activities of tertiary and industrial sectors had higher PAH concentrations in transplanted mosses, mainly for the sum of the 16 EPA-PAHs and for the 2-, 3- and 5-ringed PAHs, than areas occupied by urban and wooded areas. These PAHs were associated with enhanced Zn and Cu and land use activities that linked the sites to high traffic density. Industrial land use influences PAH concentration in water up to 1000 m of distance from the stream whereas tertiary sector land use influences up to 500m.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) include toxic organic compounds that can be found in wastewaters and sewages released from industrial and/or urban areas; these wastewaters and sewages are sometimes discharged untreated to stream waters and can be a problem to human health. Their presence in receiving waters is associated with toxicological effects, producing endocrine disruption in marine organisms (Porte et al., 2006), neurotoxicity (Tiffany-Castiglioni et al., 2006), and alterations at the ecosystem level (Chapman, 2004). Directive 2008/105/EC specifies that hazardous PAHs should be monitored in surface waters. Though PAHs occur naturally in the environment, generated by forest fires and volcanic eruptions, the largest amount of PAHs is released into the environment by human activities (Mastral et al., 2003). Anthropogenic PAHs result mainly from pyrolytic processes, especially the incomplete combustion of organic materials during industrial activities, home heating, power generation, incineration and

vehicle emissions, and as well as from petroleum cracking and refining in petrochemical industries, and during chemical manufacturing (Mastral et al., 2003).

Illegal discharges into streams are difficult to detect. Stream water concentrations only reflect very recent discharges and give little information about pollutants that may have passed through and accumulated in biota. For this reason, the use of biomonitors can be advantageous as they can accumulate pollutants over time, revealing chronic pollutant exposure. Some of the more important toxic effects of organic pollutants like PAHs, both on biota and human health, are a result of chronic exposure at very low concentrations.

Water biomonitors have been used worldwide to assess pollutants from a variety of pollution sources (metals, organic compounds, etc.). Aquatic bryophytes, specifically mosses, have proven to be effective biomonitors as they have a wide geographical and ecological distribution, lack seasonality, and are tolerant to various types of mineral and organic pollutants (Say and Whitton, 1983; Wehr et al., 1983; Roy et al., 1996). However, in some areas, particularly in more disturbed ones such as urban streams, in-situ aquatic mosses cannot be found. The use of moss transplants extends the range of sites to those without natural moss populations (Roy et al., 1996; Kelly et al., 1987; Sérgio et al., 1992; Roy et al., 1994). Moreover, moss transplants allow the calculation of enrichment factors (EFs) from natural or less contaminated sites.

Regarding specific PAHs in mosses, some studies have used transplant techniques to evaluate moss responses following exposure to these pollutants in city harbors, where levels of PAHs tend to be high and less likely to dilution effects (Roy et al., 1994, 1996). These studies have shown a strong correlation between PAH concentrations in water and PAH concentrations in moss tissues from the same sites, meaning that water pollution can be monitored using moss transplants (Roy et al., 1996). In addition, the same studies have also shown a correlation between PAH concentrations in water and adverse effects on moss physiology (Roy et al., 1994, 1996). One of the most used methods to assess the physiological status of mosses is chlorophyll fluorescence, which has becoming a valuable, non-destructive procedure to measure changes associated with photosystem II (PSII) due to gaseous pollutants and heavy metals (Maxwell and Johnson, 2000).

Though these studies reveal the potential of moss transplants to assess water pollution by PAHs, it is our understanding that no studies have used this technique to identify different pollution sources; and nowadays knowledge of the contribution of each pollutant source to stream PAH levels is needed for pollution abatement and management. In order to confirm the origin of PAHs, different chemical compounds, such as heavy metals, can be used as surrogates of specific pollution sources.

Thus, the main objective of this work was to identify the major PAH pollutant sources in urban streams using transplanted aquatic mosses and information on land-use surrounding each stream.

EXPERIMENTAL SECTION

Sampling

The aquatic bryophyte, *Fontinalis antipyretica* Hedw., was collected at a permanent natural stream located in Serra de São Mamede, Portalegre, Portugal. This species was selected because it's common and abundant in natural streams worldwide, it's easy to identify and it has been used in previous studies regarding PAHs and metal biomonitoring (Roy et al., 1996; Maxwell and Johnson, 2000). The samples were immediately transported to the laboratory in thermal boxes filled with the river water. At the laboratory, the moss was maintained in aerated commercial spring water of known composition for no more than one week at an average temperature of 15 ºC. Moss thalli were then prepared for transplantation using nylon bags with approximately 15×15 cm as proposed by Cenci (2000). Samples of approximately 100 g fresh weight (equivalent to dozens of individual thalli) were arranged inside the bag in a single layer to avoid superimposition of individual thalli. The moss bags (n=12) were then transplanted to four different streams in the Lisbon municipality of Oeiras, a densely populated urban area (Figure 1). Previous works have shown that moss transplants don´t significantly change their physiological performance when transplanted to the control site, namely regarding the ratio Fv/Fm of the chlorophyll *a* fluorescence (Sérgio et al., 1992). In this way, we assume that there is no significant physiological impact of the transplant during 3 months and thus no moss transplant was placed at the control stream.

Moss transplants were exposed for 3 months from May to August 2008, after which they were collected into glass bottles and transported to the laboratory. The bottles were kept inside cold boxes during transport and in the laboratory. At the laboratory, each moss sample (control and transplants) was divided into three parts for PAH analyses, metal analyses, and chlorophyll a fluorescence measurements.

Study area

The study was conducted in the municipality of Oeiras, Portugal (Figure 1). Oeiras is a general urban area of 3748.8 inhabitants/km^2 within the Lisbon metropolitan area. Lisbon metropolitan area, with a density of 1472.2 inhabitants/km^2, is the most densely populated area in Portugal, contrasting with the national average of 115.3 inhabitants/km^2 (INE, 2007). Oeiras main economic activities are in the tertiary sector (services): according to the data from the National Statistics Institute (INE, 2007), 80.8% of employees in Oeiras establishments work for the tertiary sector, while only 18.8% and 0.004% work for the secondary (manufacturing) and primary (involving the change of natural resources, or raw materials, into primary products) sectors, respectively. Like other urban areas in European countries, in this municipality urban areas (residential areas) can be found close to industries and to activities of the tertiary sector (Figure 1).

Land-use characterization

The geographic coordinates of each sampling site, as well as the land-use map for the study region provided by the municipality of Oeiras, were inserted into a geographic information system – ESRI® ArcMap 9.3. Using this software, buffers (with radii from 100 m to 2000 m) centered at each sampling site were drawn and the areas occupied by each land-use class that existed inside each buffer were computed. The land-use classes considered were: industrial manufacturing areas (Industrial), urban or residential areas (Urban), facility areas or areas occupied by activities of the tertiary sector (Ter) and wooded areas (Trees).

PAH analysis

All PAH analyses took place at the certified laboratory of the Portuguese Environmental Protection Agency (APA). Approximately 2 g of sample was extracted in a Soxhlet with 200 mL of acetonitrile for 24 h. After extraction, the extracts were concentrated by rotary vacuum evaporation and cleaned-up in a florisil column with 30 mL of acetonitrile as the eluting solvent. Then, the extracts were evaporated and concentrated

with a gentle stream of purified N_2 to 1 mL. The samples were analyzed by high-performance liquid chromatography (Hewlett Packard), using two columns (Agilent C18 and Phenomenex C18), coupled to an ultraviolet fluorescence detector (FLD) and to an ultraviolet/visible detector (DAD/V-UV). The sixteen US EPA-PAHs analyzed were: acenaphtylene, naphthalene, fluorene, phenanthrene, fluoranthene, chrysene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, acenaphtene, anthracene, pyrene, benzo[a]pyrene and indeno[1,2,3-cd]pyrene. Concentrations below the detection limit were assumed to be ½ of the detection limit. PAH standards of Ultrascientific with an uncertainty of 5% were used. Recovery tests using Cryptogamic organisms were performed and showed percentage values between 60±20 (for acenaphtlylene) and 107±24 (for phenanthrene).

Metal analysis

For metal analyses, 300 mg of control and transplant samples, dried at 50 ºC for one week, were subjected to digestion in 4 mL of 67% nitric acid (HNO_3). For each digestion, concentrations in acid blanks were subtracted from the transplant sample results. After the digestion, each solution (samples, blanks and standards) was separated into three replicates and diluted with 10 mL of deionised water. Elemental zinc (Zn), copper (Cu), iron (Fe), chromium (Cr), manganese (Mn) and mercury (Hg) were analyzed by atomic absorption spectroscopy (Varian Techtron AA6, United Kingdom) using an air/acetylene chamber. Elemental lead (Pb), nickel (Ni), cadmium (Cd) and cobalt (Co) were analyzed by atomic absorption spectroscopy (CBC 932 plus) with a graphite chamber (GBC GF 3000). The analytical accuracy of the results was checked against the reference material referred by the Finish Forest Research Institute, Muhos research Station (Steinnes et al., 1997). The results of the analyzed elements were within the confidence intervals of the certified values.

Measurements of chlorophyll a fluorescence

The effect of stream water pollutants on the photosynthetic capacity of the moss transplants, after 3 months of exposure, was determined from fluorescence of chlorophyll *a*. A Mini Pam 101 Chlorophyll Fluorometer (Walz, Effeltrich, Germany) was used to measure chlorophyll *a* fluorescence in three samples of *F. antipyretica* from each sampling site. All samples were dark adapted for 10 minutes in saturated humidity

before the fluorescence measurements. Dark adaptation maximizes oxidation of the primary quinine electron acceptor of PSII. After 10 minutes, the minimum fluorescence level with open PSII reaction centres (F_0) was measured by a weak red measuring beam, followed by a saturation light pulse to determine the maximum fluorescence (F_m) level with closed PSII reaction centres. Variable fluorescence, F_v, is the difference between F_m and F_0, and was calculated to obtain the parameter F_v/F_m. Chlorophyll fluorescence of control samples was also measured. It has been shown that F_v/F_m parameter is a quantitative measure of the photochemical efficiency of photosystem II (Maxwell and Johnson, 2000). The accumulation of excessive excitation energy can cause photoinhibition or photooxidation in the photosynthetic apparatus, and the reduced values of Fv/Fm indicate that a proportion of PSII reaction centres are damaged (Maxwell and Johnson, 2000).

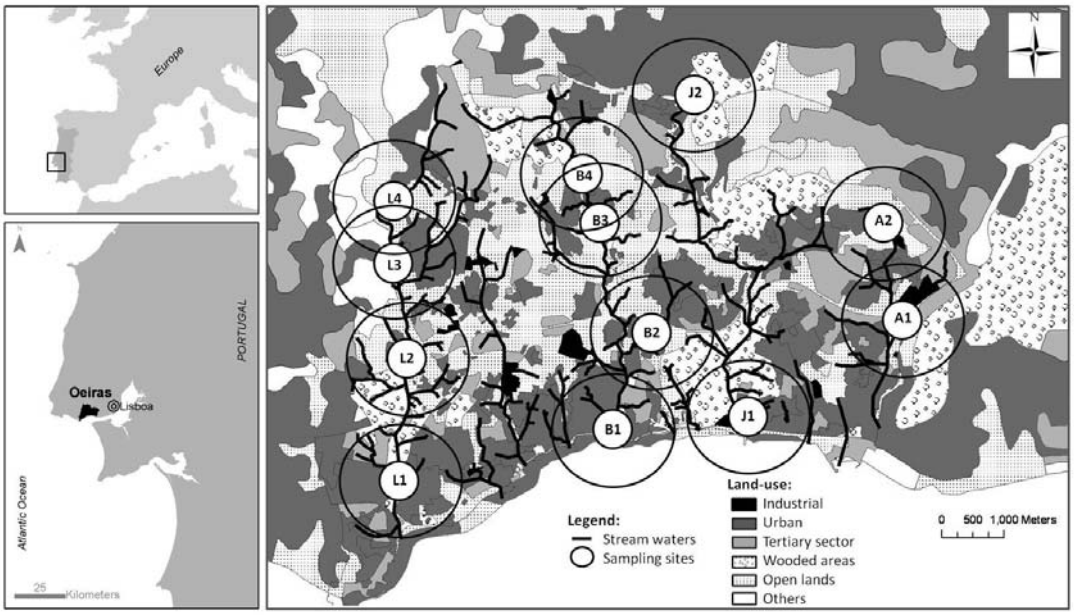

Figure 1. Location of sampling sites in streams of the densely populated area of Oeiras, in Lisbon, Portugal, where moss transplants were exposed for 3 months (n=12). One kilometre buffers are represented in the figure by circles. The study area faces Tagus River (one of the main rivers of the country) in the south band.

Statistical analysis

Pearson linear correlation coefficients between PAH or metal concentrations and chlorophyll fluorescence in the moss samples were calculated. Pearson linear correlation coefficients were also calculated for the correlation between PAH concentrations and the area covered by each class of the land-use at each sampling site within buffers of increasing radii, from 100 m to 2000 m (method adapted from (Pinho et al., 2008). The coefficient (R-value) obtained for each correlation was plotted against the buffer radii. The land-use classes considered were Industrial, Urban, Tertiary, and Trees. A p-value < 0.05 was considered significant.

RESULTS AND DISCUSSION

Accumulation of PAHs in aquatic mosses transplanted to urban streams

The accumulation of PAHs in mosses collected from a natural stream and transplanted to urban streams could be evaluated in the study area using enrichment factors (EFs). The EF for any individual pollutant is the ratio of the concentration of that pollutant in the transplanted moss to concentration in the control moss. In general, transplanted aquatic mosses accumulated higher concentrations of the high molecular weight compounds (HMW), namely 5- and 6-ring PAHs, as shown in Figure 2. EFs for the 5-ring PAHs ranged from 2 to 4; EFs for 6-ring PAHs ranged from 5 to10. Note that HMW EFs were highly variable (Figure 2). EFs for 2- and 3-ring PAHs were close to 1.0, although some extreme EFs were noted among the 2-ring PAHs. Concentrations of 4-ring PAHs in moss transplants were also only slightly higher than concentrations in control mosses from the natural area—the median EF did not reach 2 (Figure 2).

These results suggest that, in general, urban environments are not an important source of 2-, 3- and 4- ring PAHs to the stream waters, but do tend to be a source of 5- and 6-ring PAHs and occasionally of 2-ring PAHs. While the LMW PAHs are more related to industrial activities, several authors have reported an increase of the HMW PAHs in atmospheric deposition in urban areas, as a consequence of fossil fuel combustion emissions, particularly from vehicles (Augusto et al., 2009; Srogi, 2007). Although the 6-ring PAHs showed higher EFs when compared to the 5-ring PAHs, it is difficult to distinguish the origin of each group, as usually emission sources emit both groups of pollutants (5- and 6-ring PAHs) at the same time. In this way, 5- and 6-ring PAHs are

both associated with combustion process – they both have a pyrogenic origin. Atmospheric deposition includes wet and dry deposition of particles and vapor. PAHs exist in both gaseous and particulate phases in air, and are also subjected to washout from the atmosphere (Srogi, 2007).

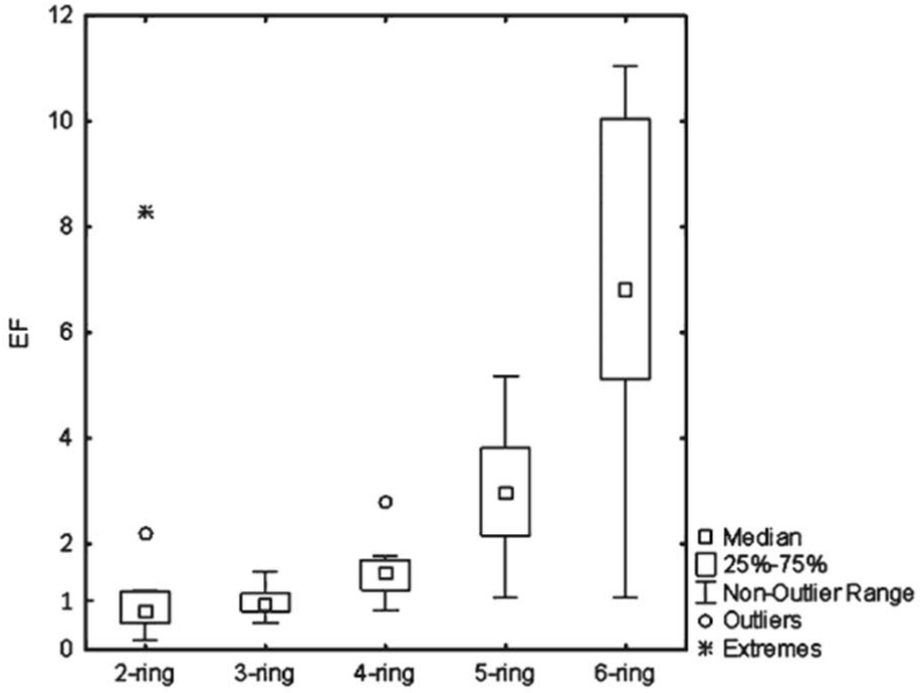

Figure 2. Enrichment factors (EF) of PAHs classified by ring number in the aquatic moss *Fontinalis antipyretica*, transplanted for 3 months to urban streams. The enrichment factors were obtained comparing the concentrations of PAHs prior to transplantation with the ones after the exposure period (n = 12).

The high variability of 5- and 6-ring PAH concentrations in mosses from urban streams, indicates that input of these compounds must depend on the presence of specific emission sources located at certain areas, showing the need for further land-use analysis. To allow a detailed analysis regarding this type of contamination, enrichment factors at each sampling site are presented (Table 1).

The EF for each of the 16 EPA-PAHs, and for PAHs classed by the number of rings in their molecular structure, by sampling site, are presented in Table 1. PAHs in mosses from stream A at sampling site A1 were most enriched compared to the control sample.

After three months of exposure, the sum of the 16 EPA-PAHs concentrations in these transplants was enriched more than three fold. Enrichment of the 16 EPA-PAH (EF > 1.5) also occurred at both B4 and J2. In contrast, the sum of the 16 EPA-PAHs decreased slightly in mosses transplanted to L3 and B2 in relation to control samples (Table 1).

TABLE 1. Enrichment factors for transplanted mosses exposed for 3 months in the four streams (A, B, J and L) located in the highly urbanized study area. Enrichments above 3.00 are in bold for both different ring-compounds and PAH congeners. Numbers between parentheses refer to the number of rings.

	Stream A		Stream B				Stream J		Stream L			
	A1	A2	B1	B2	B3	B4	J1	J2	L1	L2	L3	L4
2-ring PAHs	**8.29**	0.79	0.39	0.47	1.08	0.66	0.19	2.20	0.93	1.12	0.54	0.62
3-ring PAHs	1.49	0.78	0.70	0.94	0.81	1.29	0.50	0.75	0.91	1.06	0.57	1.06
4-ring PAHs	1.63	1.41	1.76	0.75	1.52	2.80	1.50	1.77	1.09	1.38	1.01	1.18
5-ring PAHs	**5.45**	**6.54**	**3.36**	2.75	**4.74**	**6.91**	**6.59**	**4.62**	**3.31**	**4.12**	1.00	**4.21**
6-ring PAHs	**11.06**	**10.29**	**6.78**	**5.02**	**6.84**	**10.31**	**8.25**	**9.82**	**4.90**	**5.22**	1.00	**5.42**
Acenaphtylene (3)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Naphthalene (2)	**10.79**	0.93	0.39	0.48	1.25	0.62	0.16	2.91	1.09	1.26	0.55	0.66
Fluorene (3)	2.42	0.46	0.39	0.46	0.68	0.77	0.26	0.54	0.56	0.78	0.52	0.54
Phenanthrene (3)	1.35	0.75	0.64	0.93	0.78	1.29	0.44	0.69	0.90	1.07	0.51	1.07
Fluoranthene (4)	2.65	2.24	2.72	1.17	1.72	**3.59**	2.25	**3.09**	1.65	2.07	0.96	1.88
Chrysene (4)	**5.06**	**6.57**	**5.90**	2.54	**4.09**	**11.57**	**5.92**	**6.27**	2.94	**3.15**	1.00	**5.09**
Benzo[a]anthracene (4)	**6.84**	**5.61**	**9.90**	**4.78**	**13.98**	**13.87**	**11.03**	**3.72**	**4.26**	**4.96**	1.00	**4.85**
Benzo[b]fluoranthene (5)	**8.34**	**8.47**	1.00	**4.41**	**6.99**	**10.16**	**11.18**	**8.04**	**5.50**	**5.93**	1.00	**7.47**
Benzo[k]fluoranthene (5)	**6.00**	**5.56**	1.00	2.46	**4.89**	**6.97**	**6.86**	**3.72**	**3.23**	**4.11**	1.00	**4.06**
Dibenzo[a,h]anthracene (5)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Benzo[g,h,i]perylene (6)	**16.59**	**19.58**	**12.55**	**9.04**	**12.67**	**19.62**	**15.50**	**18.64**	**8.81**	**9.44**	1.00	**9.84**
Acenaphtene (3)	**7.22**	1.00	1.00	1.00	0.83	1.73	1.00	1.95	1.00	1.00	1.00	1.00
Anthracene (3)	**3.37**	1.12	1.97	1.00	1.47	1.85	1.06	1.28	1.00	1.00	1.00	1.04
Pyrene (4)	0.93	0.73	0.92	0.37	0.82	1.70	0.67	1.05	0.68	0.92	1.03	0.62
Benzo[a]pyrene (5)	**6.47**	**11.11**	**10.45**	**3.14**	**6.08**	**9.52**	**7.33**	**5.73**	**3.52**	**5.44**	1.00	**4.30**
Indeno[1,2,3-cd]pyrene (6)	**5.53**	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Σ 16 EPA-PAHs	**3.35**	1.34	1.14	0.92	1.32	1.95	1.08	1.67	1.12	1.35	0.71	1.18

Because PAH profile differed among sampling sites within the same stream; most probably the pollutants have a more local origin and are quickly diluted by stream water or deposited to sediments. This is very clear from the observation of PAHs in samples B2 and L3 which are in the middle of other sites that were shown to be more polluted downstream and upstream. As PAHs are lipophilic compounds, they tend to adhere to organic matter and suffer bioaccumulation in biota. This will be further discussed in relation with the land-use and distance of impact.

Results also showed that the enrichment of 6-ring PAHs by mosses transplanted to stream A is particularly high, being 10 fold more concentrated than the control site (Table 1). Mosses from site A1, were exceptionally enriched in the 2-ring PAHs (8-fold more than controls) in contrast to all other transplanted mosses, which had enrichment factors between 0.19 and 2.20 (Table 1). The high value for 2-ring PAHs at site A1 is mainly due to naphthalene, which suggests that an industrial or petrol station discharge might have occurred into this stream during the exposure period. After a discharge into a stream, pollutants such as PAHs and the majority of metals tend to become diluted in the water and to accumulate in the sediments; therefore, water analyses usually provide values that are below detection limits and sediment analyses usually reflect long-term deposition of pollutants (Meador et al., 1995). For these reasons, illegal discharges are very difficult to observe and monitor using water because it reflects only short-term pollution episodes or sediment analyses because they accumulate historical pollution episodes.

The use of biological organisms that retain pollutants in their tissues might be a way of monitoring water pollution, integrating more time than a water analysis but less than a sediment one. Transplants are very flexible since we can use it for 3 months or more and obtain with this a rate of deposition since we are having in account the time dimension that is difficult to obtain with in situ organisms or sediment. Aquatic mosses tend to accumulate pollutants in their tissues during the exposure period and if any discharge is made during this period, mosses will probably reflect this contamination signal. Moreover, the accumulation of pollutants in aquatic macrophytes (as for example aquatic mosses) shows an evidence of PAH bioaccumulation in plant material that will feed other living-organisms and contaminate the food-chain.

The specific contamination of the sampling site A1 supports our conclusion that contamination can be quite local and that, in as little as 3 months, mosses are able to integrate a profile of compounds to which they have been exposed. In terms of public health, the fact that some stream waters could be contaminated with such carcinogenic and mutagenic compounds is very important, because most water analyses are generally under detection limits for these kinds of contaminants and thus this methodology could work to detect localized water contamination.

PAHs in relation to the land-use and metals

Tertiary sector (service) and industrial areas showed the highest correlation coefficients between land-use and PAH concentrations (Figure 3). This is true for the sum of the 16 EPA-PAHs and also for 2-, 3- and 5-ring PAHs (Figure 3). Industrial areas had a significant effect if they were located less than 1000 m from streams (radii for which R-values were greater), whereas areas occupied by the tertiary sector had a significant effect if they were located less than 500 m from streams (Figure 3).

To detect sources of PAHs in stream waters, correlations were calculated between PAH and metal concentrations in the moss transplants. Results are presented in Table 2. Sums of the 16 EPA-PAHs and the 2, 3 and 5-ring PAHs were significantly positively correlated with both Zn and Cu. This means that higher input of PAHs (especially of 2, 3 and 5-ring PAHs) is related to sources that also emit Zn and Cu. Urban runoff water quality problems are caused by the cumulative effects of many sources including heavy and light industry, road runoff and spills, and illegal dumping (Walker et al., 1999). The most significant sources of Zn urban runoff include atmospheric fallout, corrosion, tires, pavement water, automobile exhausts, exterior paint, road salt, and other terrestrial sources (US Government, 2001). The sources of Cu include corrosion of copper plumbing, electroplating waste, some algaecides, brake linings, and asphalt pavement wear (Walker et al., 1999). The US EPA highlights 21 toxic substances that can mainly be assigned to road traffic; some heavy metals, such as Cu, Zn, are among them (US Government, 2001).

It is important to remember that the main economic activities present in the study area are in the service sector; 80.8 % of the employees in this area work for the tertiary sector, while only 18.8 % and 0.004 % work for the secondary and for the primary sectors, respectively (INE, 2007). Associated with the tertiary sector are a set of roads with a considerable traffic flow, which might be responsible for the input of PAHs into the streams, as corroborated by the correlation between concentrations of the 16 EPA-PAHs and concentrations of Cu and Zn in the transplanted mosses (Table 2). The 2-, 3- and 5-ring PAHs also are positively correlated with these same elements (Table 2). As previously reported, urban runoff contains PAHs deposited on surfaces, as well as mobile related PAHs from gasoline and oil drips and spills, exhaust products, tire particles, and bitumen from road surfaces (Srogi, 2007).

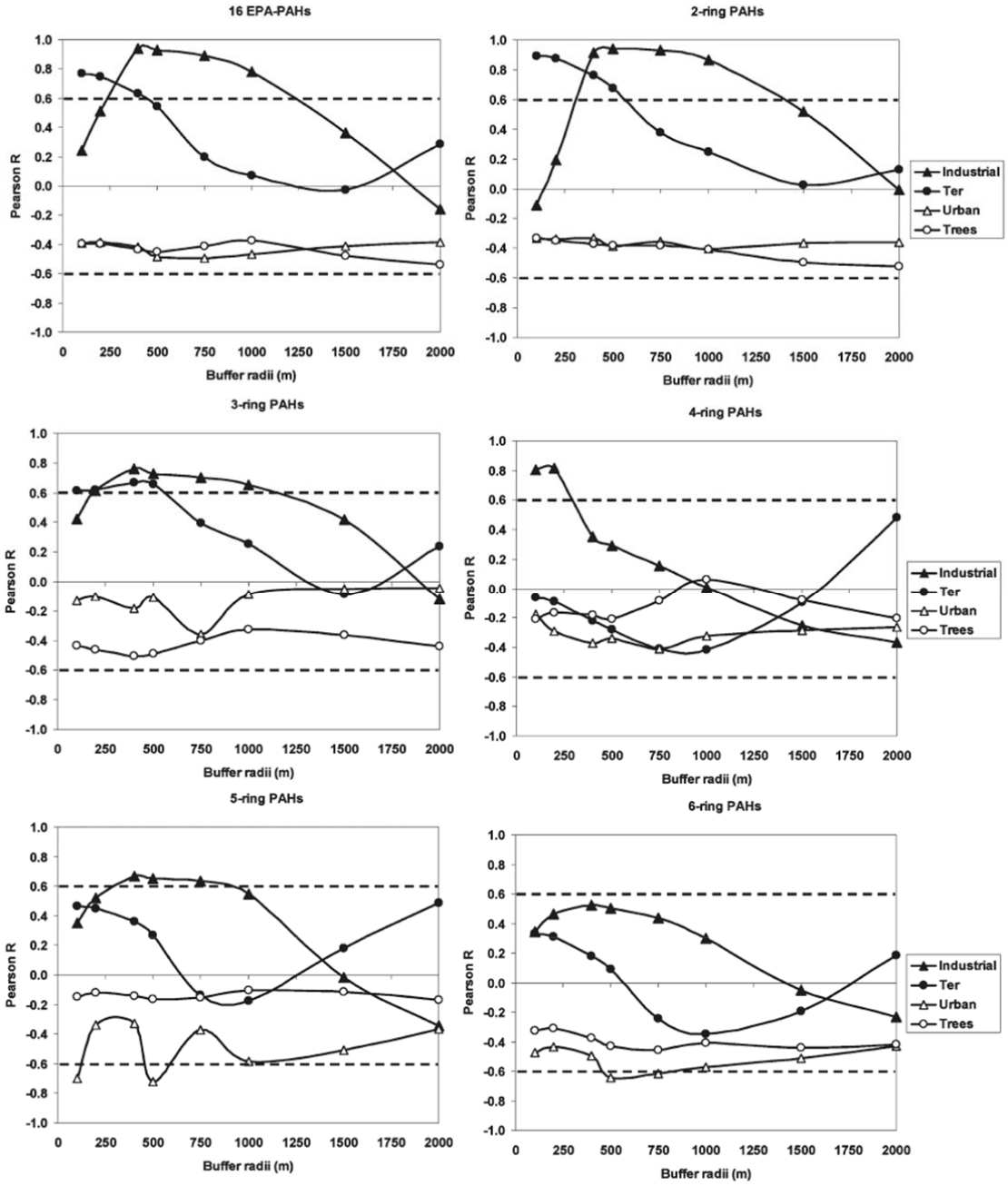

Figure 3. Pearson's correlation coefficients (R) for the area occupied by each land-cover type (industrial, tertiary activities, urban (residential), and wooded areas-Trees) and the PAHs concentrations by ring number. The areas above the upper and below the lower dashed lines represent values with significant R values ($p<0.05$).

The main anthropogenic sources of PAHs in stream waters of our study area seem to be most likely related to traffic and urban-industrial pollution. We suggest that enrichment of the HMW compounds in all transplanted mosses (Figure 2 and Table 1) reflects urban runoff, in accordance with other authors who demonstrated a relation between these PAHs and urban runoff and showed that the influence of traffic pollution appears to be reduced if the distance from a road is > 500 m (Walker et al., 1999).

TABLE 2. Pearson's coefficients for 1) correlations between PAH and metal concentrations and 2) PAH concentrations and photosynthetic capacity, F_v/F_M, measured in aquatic mosses transplanted to urban streams for 3 months (n=12). Significant correlations are shaded ($p<0.05$).

	Zn	Cu	Fe	Pb	Mn	Ni	Cr	Co	Hg	Cd	F_v/F_M
2-ring PAHs	**0.65**	**0.91**	-0.39	-0.31	0.06	-0.10	-0.21	-0.36	-0.13	-0.18	0.15
3-ring PAHs	**0.66**	**0.60**	-0.34	-0.23	-0.15	-0.10	0.01	-0.12	0.28	0.08	0.05
4-ring PAHs	0.40	0.15	0.39	0.25	0.00	0.06	0.51	-0.57	-0.26	-0.24	0.10
5-ring PAHs	**0.65**	**0.70**	0.09	-0.06	-0.18	-0.23	0.22	**-0.75**	-0.12	-0.35	0.07
6-ring PAHs	0.50	0.57	0.19	0.26	0.08	0.03	0.26	**-0.79**	-0.33	-0.49	0.24
Acenaphtylene (3)	**0.68**	**0.91**	-0.54	-0.35	-0.08	-0.19	-0.28	-0.13	0.01	0.17	-0.06
Naphthalene (2)	0.54	0.52	-0.49	-0.30	-0.10	-0.21	-0.18	0.16	0.00	0.46	-0.42
Fluorene (3)	0.52	**0.59**	**-0.58**	-0.32	-0.14	-0.26	-0.27	0.18	0.00	0.36	-0.39
Phenanthrene (3)	**0.66**	**0.90**	-0.57	-0.43	-0.10	-0.29	-0.34	-0.26	-0.09	-0.04	-0.11
Fluoranthene (4)	**0.58**	**0.75**	**-0.65**	-0.56	-0.23	-0.45	-0.39	-0.13	-0.19	0.02	-0.38
Chrysene (4)	0.36	**0.64**	**-0.79**	-0.46	-0.11	-0.36	**-0.58**	0.00	-0.09	0.18	-0.32
Benzo[a]anthracene (4)	**0.62**	**0.89**	-0.52	-0.43	-0.17	-0.32	-0.31	-0.27	-0.14	-0.14	-0.09
Benzo[b]fluoranthene (5)	0.57	**0.80**	**-0.66**	-0.42	-0.20	-0.33	-0.34	-0.01	-0.14	0.05	-0.29
Benzo[k]fluoranthene (5)	**0.60**	**0.79**	**-0.63**	-0.39	-0.15	-0.30	-0.34	-0.02	-0.10	0.16	-0.28
Dibenzo[a,h]anthracene (5)	0.51	0.55	**-0.60**	-0.47	-0.31	-0.48	-0.26	0.05	-0.26	0.02	**-0.59**
Benzo[g,h,i]perylene (6)	**0.71**	**0.88**	-0.52	-0.45	-0.23	-0.36	-0.24	-0.16	-0.10	-0.04	-0.25
Acenaphtene (3)	**0.59**	**0.72**	**-0.60**	-0.43	-0.14	-0.35	-0.33	-0.10	-0.11	0.17	-0.34
Anthracene (3)	**0.67**	**0.89**	-0.57	-0.44	-0.17	-0.32	-0.31	-0.20	-0.11	0.00	-0.17
Pyrene (4)	**0.66**	**0.88**	**-0.59**	-0.45	-0.16	-0.33	-0.33	-0.18	-0.10	0.05	-0.20
Benzo[a]pyrene (5)	**0.64**	**0.78**	-0.53	-0.28	-0.02	-0.12	-0.25	0.05	0.10	0.43	-0.14
Indeno[1,2,3-cd]pyrene (6)	**0.65**	**0.88**	-0.53	-0.44	-0.19	-0.34	-0.29	-0.24	-0.15	-0.08	-0.16
Σ 16 EPA-PAHs	**0.75**	**0.87**	-0.21	-0.17	0.00	-0.09	0.02	-0.53	-0.14	-0.23	0.16

Impact of PAHs on the photosynthetic capacity of aquatic mosses transplanted to urban streams

Chlorophyll fluorescence measurements were performed to evaluate the impact of pollutants present on the stream waters in the photosynthetic capacity of the mosses after three months of exposure. The F_v/F_M parameter measured in the transplanted mosses was generally low (between 0.06 and 0.57, which correspond to 8.6 and 80 % of

the control) compared to control samples (0.70). Some reduction of the F_v/F_M parameter was expected, as the mosses were transplanted to a different place and thus had been subjected to different ecological conditions. The lack of significant linear Pearson's correlations between moss PAH concentrations and the F_v/F_M parameter in the same transplants indicates that the PAHs in the streams were not the most important pollutants causing damage to the photosynthetic apparatus of the aquatic moss transplants (Table 2). An exception was dibenzo[a,h]antracene, which had a significant negative correlation with F_v/F_M, indicating that this compound could have influenced moss physiology (Table 2). Moss transplants are subjected to a wide range of pollutants and other non-favorable ecological conditions in those urban stream waters, other than PAHs. This kind of measure is useful as it integrates the effect of all pollutants (known and unknown), including the synergistic and antagonistic effects and provides an estimate of pollution effects on plant biota.

This study showed for the first time that urban streams seem to have a scattered contamination HMW-PAH; the areas occupied by tertiary (services) and industrial sectors have greater effects on moss transplants to urban streams, mainly for the sum of the 16 EPA-PAHs and for the 2-, 3- and 5-PAHs, than other urban areas. These PAHs were correlated with sources that also emit Zn and Cu, which suggests a traffic-related origin. Industrial areas were most likely to be associated with enriched PAH concentrations in moss transplants if they were located < 1000 m from the streams, whereas tertiary sector areas were most likely to be associated with moss PAH enrichment if they were < 500 m away. Thus, it is advisable to locate both industry and roads away from stream waters using the previous distances. In addition, as this technique is economically and practically feasible, it can be easily used by water monitoring agencies, allowing to control PAH concentrations in water and also to identify pollution sources.

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Chapter 04 |

Assessing environmental and human health risk based on integration of different monitoring approaches

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4.1 | The contribution of environmental biomonitoring with lichens to assess human exposure to dioxins

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ABSTRACT

The contribution of environmental biomonitoring with lichens to assess human exposure to dioxins was the main purpose of this work. For that, PCDD/Fs were measured in 66 lichen sampling points. The obtained information significantly improved the basic knowledge on the environmental exposure to dioxins through: distinction between effective control areas from areas with moderate atmospheric deposition; integration of PCDD/F atmospheric deposition for much longer periods, allowing to relate low levels with long-term chronic effects on health; production of high resolution data on environmental exposure essential to perform reliable environment-health studies. It was argued that PCDD/Fs in lichens may be used as spatial estimators of the potential risk of inhalation by the population present in the area. An example of the application of this data to select control and exposed areas for environmental health studies was presented.

INTRODUCTION

Polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (PCDD/Fs) are organic compounds formed as unwanted by-products of combustion in many industrial chemical processes, and have been detected in almost all environmental matrices: soil, sediment, air, water, animals, vegetation (Rappe 1993, WHO 1992, Fiedler et al. 1990). These compounds are very important in environmental-health studies because they are carcinogenic and potentially toxic, even in very low concentrations. Atmosphere is a major pathway for transport and deposition of PCDD/Fs on different environmental media.

Human health risk assessment requires identification of the pathways through which people can be potentially exposed to these chemicals (Meneses et al., 2004). The quantitative estimation of health risk due to an environmental exposure can be considered as a combination of five pathways, namely: air inhalation, foodstuffs (including breast feeding), drinking water, absorption through skin and soil ingestion (Meneses et al., 2004). Although the main route of PCDD/F human contamination is through food ingestion, for an accurate health risk estimate, it is essential to evaluate every other exposure route; hence, the pollutant concentrations in each environmental media must be determined (Nessel et al., 1991).

In epidemiological studies, the environmental observations must be made in a way that reflects as closely as possible the exposure of the population under observation. Since such a level of information is difficult to attain, many studies are based on estimated exposure, from data obtained at monitoring sites, previously selected for regulatory purposes rather than for estimating the real exposure of the population (EHC 27, 1983). Sampling sites tend to be selected for their expected relatively high concentrations and are often placed at a much higher altitude than the human breathing zone (EHC 27, 1983).

The most acceptable way to perform an accurate measurement of the environmental level of a particular pollutant is through a biomonitoring program (EHC 27, 1983). Environment biomonitoring consists in the monitoring of the pollutant levels in the environment by means of living organisms (Martin and Coughtrey, 1982; Puckett, 1988; Sloff, 1993). Lichens, the most studied biomonitors of air pollution, are symbiotic organisms consisting of fungi and algae or cyanobacteria. Many authors have pointed out that lichens have several of the characteristics required for the ideal biological monitor (Manning and Feder, 1980; Martin and Coughtrey, 1982; Nimis, 1990; Garty, 1993; Sloof, 1993; Branquinho 1997, 2001). They have been used because they are sensitive to variations in environmental conditions with a change in their "signs of activity" (Markert et al., 1997). These changes may occur: i) in the structure or dynamics of the population or community of organisms; ii) in changes in their functional response, which may lead to changes in vitality; iii) in concentrations of elements which influence the organism (Branquinho, 2001).

Lichens have been used to biomonitor several pollutants levels, particularly of sulphur, nitrogen, fluoride, oxygen, metals, radionuclide, dioxins, other organic compounds, etc. (Martin and Coughtrey, 1982; Puckett, 1988; Garty, 1993; Augusto et al., 2004a,b,c). The concentrations of given pollutants, measured within the organism, are used to reconstruct the spatial and temporal deposition patterns of the pollutants deposited at a location. As a result, lichens have been very helpful to identify several anthropogenic sources such as roads, mines and industrial facilities in urban and rural locations (Branquinho, 2001), and natural sources, such as volcanoes.

Recent work on the performance of lichens as biomonitors of organic compounds, particularly PCDD/Fs, has shown the potential of these organisms for monitoring PCDD/Fs atmospheric deposition (Augusto et al., 2004a,b,c). The goal of this study is to

show how environmental data obtained through biomonitoring with lichens can be integrated in environmental health studies.

EXPERIMENTAL SECTION

Lichen sampling

The area studied was Setúbal peninsula, located in south Portugal, covering an area of 150 000 ha. The lichen species selected for biomonitoring of PCDD/Fs atmospheric deposition was *Xanthoria parietina* (L.) Th. Fr. growing on house roof-tiles (Augusto et al., 2004a). Lichen sampling was performed during March 2000, after a dry period of 84 days (precipitation below 7 mm), during a meteorological stable period, at 66 locations.

PCDD/F analysis

After collection, the lichens were stored in plastic bags and transported to the laboratory, where the unwashed samples were immediately dried at room temperature and sorted to remove extraneous material (other lichen or moss species). The cleaned samples (*c*. 15 g) were then ground (Glen Creston Ltd. MM 2000) and analysed for PCDD/Fs concentration. Ground and dried lichen samples were added with labelled standards $^{13}C_{12}$, subjected to toluene extraction and purified to remove intrusive substances. PCDD/Fs were quantified by gaseous chromatography and high-resolution mass spectrometry (Fisons Autospec Ultima System). The precision and accuracy of the analysis was checked against reference material. Toxicity of each sample was determined through the sum of the concentrations (ng I-TEQ Kg^{-1}) calculated for the toxic congeners.

Demographic indicators

The demographic indicators “resident population” and “resident population under 14 years old”, published by National authority in statistics (INE, 2006), at the parish level were used for the following investigation.

Mapping environmental and demographic indicators

The PCDD/Fs concentrations measured in lichens samples were assumed as indicators of PCDD/Fs atmospheric deposition levels. This data set shows a positively skewed distribution. The experimental semivariograms were calculated and, as they did not show any preferential direction of spatial dispersion, an isotropic semivariogram model

comprising two spherical structures was fitted with ranges of 6 and 26 km, respectively. Based on the fitted semi variogram model, a map of PCDD/Fs atmospheric deposition levels was estimated using ordinary kriging (geoestatistical estimator) on a regular grid of 310 x 200 nodes with a grid spacing of 200 m. To perform all the geostatistical calculations the program geoMS – Geostatistical Modelling Software (CMRP-IST, 2000) was used. Then, using ArcGIS Desktop 9.1 (ESRI INC, 1999-2005) a geographical information system was built gathering all data together, namely, parish limits, demographic data and environmental data, in the same geographical referential. Afterwards, using geographical information systems tools, PCDD/Fs atmospheric deposition levels statistics at a parish level were calculated, such as means and standard deviations, obtaining a set of maps to which demographic indicators were overlapped, for helping in interpretation and results discussion.

RESULTS AND DISCUSSION

Environmental assessment of exposure to dioxins

In order to assess environmental exposure to dioxins, data obtained through the chemical analysis of lichens collected in the region under study was processed by means of geostatistical methodologies. Thus, the production of high resolution maps with important information regarding the deposition of these compounds in the region significantly improved the basic knowledge on the environmental exposure which is critical for an adequate study of the impact of PCDD/F on human health.

Environmental health studies devise several questions: which populations are (or have been) exposed to the pollutants?; which populations should be selected for further human biomonitoring studies?; and which should be considered as control populations? To answer those questions it is necessary, firstly, to develop a monitoring program of the environmental levels of the pollutants under study (EHC 27, 1983). In air quality monitoring programs some details should be kept in mind: pollutants discharged to the atmosphere are not constant in space and time. Usually, air quality data are either sparse in space or reflect short periods of time (in the order of seconds or minutes). Furthermore, in the case of PCDD/Fs there are no monitoring stations continuously measuring the levels of these pollutants.

When compared to other environmental biomonitors or monitoring stations, lichens integrate much longer periods of pollutants atmospheric deposition (from months up to 5 years) (Coutinho et al., 1999; Branquinho, 2001; Simonetti, 2000). This kind of data is more appropriate for comparisons with health data than the short-term measurements normally performed in the air or even in plants, which report from few hours to days or to a season, respectively. In this way, lichens provide a sample of the complex mixture of PCDD/Fs that humans and biota have been exposed to in a long-term. This will be of critical importance for health studies, since one of the most difficult tasks is to relate the low pollution levels with long-term chronic effects on health (Mukerjee and Cleverly, 1987). Moreover, lichens act as simple Biological Models for pollutants deposition and effects, both on human and ecosystems. For the case of health in humans, this was shown by the work of Cislaghi and Nimis (1997), where lichens diversity used as bioindicator of atmospheric pollution showed a good correlation with lung-cancer in NE Italy.

Concerning the spatial coverage of physical monitoring networks, a single site may be assumed to represent a large area and the number of sites is often limited because of operative and financial constraints related to the installation of a monitoring station. Lichens allow the adoption of cost-effective sampling strategies with relatively high density of sampling locations, thus generating more spatially detailed data in order to obtain high resolution maps. They have a wide geographical distribution allowing comparison of pollutant concentrations from diverse regions. The maps produced in this work represent the only study of PCDD/F deposition in Portugal with high spatial resolution and consequently a reliable spatial model. These results have shown that the spatial continuity for PCDD/F deposition has a two-structure semivariogram with 6 and 26 km ranges and that the deposition of PCDD/Fs occurs both in urban and industrial areas (Augusto et al., 2004a).

On the other hand, levels of PCDD/Fs in the air measurements are frequently below detection limit, especially in background areas. In this work, all lichen samples were analysed and detectable PCDD/Fs levels were measured, even in those samples located at the less contaminated areas (ranging from 0.87 to 22.58 ng I-TEQ Kg^{-1}). In fact, lichens usually present the highest accumulation of pollutants from the environment in comparison with other living organisms because they are slow-growing, long-lived organisms. This ability of lichens to accumulate PCDD/Fs is an important advantage of

using lichens as environment biomonitors for human monitoring purposes (Augusto et al., 2004a,b,c). Since these pollutants may affect health even in very low concentrations and bioaccumulate in humans (Boening, 1998; Sweetman et al., 2000; Kogevinas, 2001; Birnbaum and Cummings, 2002), this ability to discriminate very small local spatial variability in atmospheric deposition of PCDD/Fs may be determinant in distinguishing effective control areas from areas with moderate PCDD/Fs levels (Figure 1). In the map of figure 1 small neighbour parishes of the same municipality have shown that the levels of atmospheric PCDD/Fs may differ significantly between them. This implies that populations living there are exposed to contrasting PCDD/Fs environments through atmospheric pollution, consequently only a high resolution environment exposure data set is acceptable to perform reliable environment-health studies.

Mapping PCDD/Fs inhalation risk estimated using biomonitors

In general, food intake is usually considered the primary source of exposure to PCDD/Fs and inhalation and dermal contact are only consider minor routes (Karademir, 2004; Meneses et al., 2004). The level of PCDD/Fs in food is not a variable related to the local environmental exposure in urban areas, since the populations, in general, tend to consume food from other places. In a prevention context, local decision makers find it difficult to control and regulate potential sources of contamination in non-local foodstuffs. Thus, the impact of local or regional PCDD/Fs pollution in air and its deposition on the different media has direct and indirect effects on local human health which should not be negligible. This pathway includes PCDD/F inhalation, dermal contact, soil and dust ingestion, and local food intake. In fact, results of daily intake of PCDD/Fs only by inhaling air suggested that the inhalation exposure of PCDD/Fs by the inhabitants in Liwan district is relatively high (Yu et al., 2006). Additionally, prevention measures to reduce the human exposure due to local or regional pollution is much easier to implement than to control and reduce contamination from general food intake.

In several studies, inhalation exposure is calculated by assuming that individuals were exposed to polluted air 24 h/day and that indoor air exposure was equal to outdoor exposure (Schuhmacher and Domingo, 2006; Yu et al., 2006). These measures directly reflect the concentration and profile of PCDD/F in air and are only weighted by the different ventilation rate of adults and children (Schuhmacher and Domingo, 2006; Yu et al., 2006). Pollutants (heavy metals, radionuclides, etc.) in lichens have been shown to reflect atmospheric pollution (Garty, 2001). Calibrations between the concentrations of

pollutants in lichens and those of monitoring stations have been achieved for a series of pollutants (Branquinho et al., 1998, 2004). In the same way, the PCDD/F congener and homologue profiles in lichens were compared to that of the air and soil, and it was found that PCDD/Fs in lichens were reflecting the air profile (Augusto et al., 2004a). However calibration is not yet possible due to the lack of PCDD/F monitoring stations.

Lichens are poikilohydric organisms, lacking root system and a developed cuticle, with very limited control of the uptake and loss of water and solutes from atmospheric deposition. Consequently, they developed other strategies to intercept nutrients from the air, becoming very efficient at it, and reflecting in this way the atmospheric composition. Given the reasons mentioned before, in this work PCDD/Fs in lichens were considered as spatial estimators of the potential risk of inhalation by the population resident in the same area (Figure 1 and Figure 3). Further studies, both human biomonitoring and/or epidemiological, may now be performed in order to evaluate the impact of the levels of inhaled dioxins on the total level of PCDD/Fs in the population. For that, control and exposed areas must be selected. Information such as the one presented in figures 1 and 2 may assist this decision. Figure 1 gives information about the mean concentration of PCDD/Fs in TEQ´s per parish overlaying the number of resident population in the same territory unit, whereas figure 2 gives information on the level of variance associated with each territory unit.

Since the lichens sampling in space was regularly distributed, lower standard deviation for a parish reflects consistency between the concentrations found at the sampling locations within the parish, showing that the risk of exposure to inhalation is similar all over the same unit (Figure 2). In the same way, higher standard deviations show high variability of exposure to inhalation at the same territory unit. A higher number of resident populations is also required to perform representative human health studies. Moreover, in the decision-making process concerning public health, it is advantageous to focus there where the greatest numbers of exposed persons are. In this way, priority should be given to the most contaminated areas where there is simultaneously a greater number of residents.

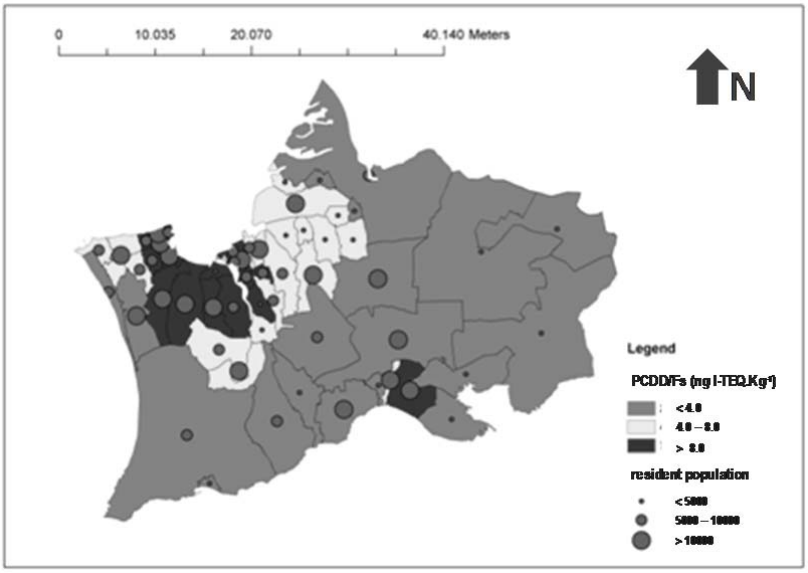

Figure 1. Spatial distribution of PCDD/F concentrations (ng I-TEQ Kg^{-1}) obtained through chemical analysis of lichen samples collected in Setúbal peninsula, Portugal, in relation to the demographic indicator "resident population" by parish. PCDD/F concentrations increase gradually from light to dark colours and the number of residents increases gradually from small to large circles.

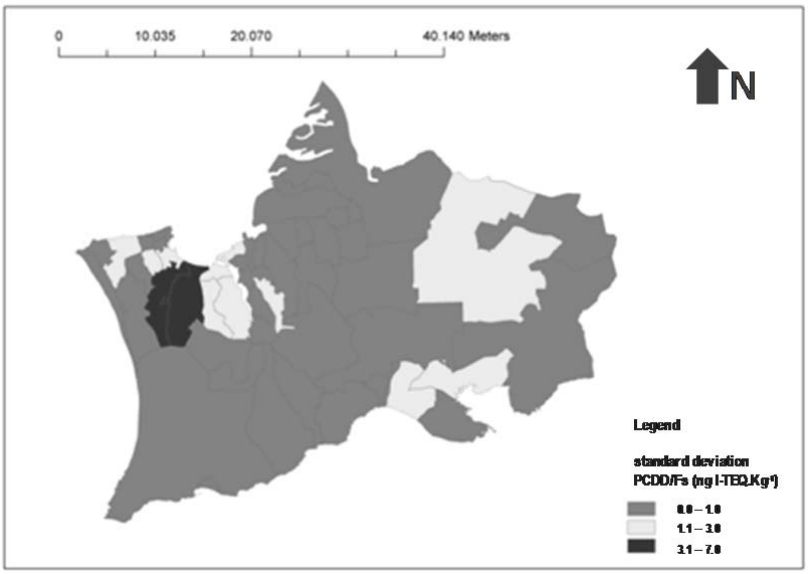

Figure 2. Spatial distribution of standard deviations of the PCDD/F concentrations by parish (ng I-TEQ Kg^{-1}) obtained through chemical analysis of lichen samples collected in Setúbal peninsula, Portugal. Variability increases gradually from light to dark colours.

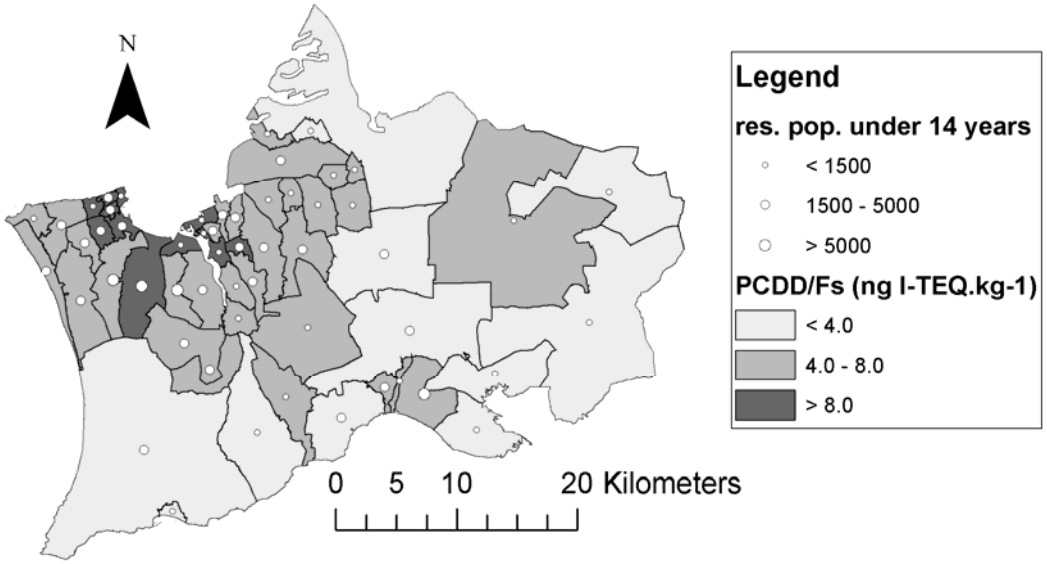

Figure 3. Spatial distribution of PCDD/F concentrations by parish (ng I-TEQ Kg^{-1}) obtained through chemical analysis of lichen samples collected in Setúbal peninsula, Portugal, in relation to the demographic indicator "infant population". PCDD/F concentrations increase gradually from light to dark colours and the number of residents increases gradually from small to large circles.

The number of residents under 14 years can be considered as a risk group regarding PCDD/Fs, since they are more vulnerable. Children are closer to the soil and are thus more exposed to ingestion and inhalation of soil particles. Although most of the information available on potential age-related differences in the toxicity of PCDD/Fs comes from experiments in laboratory animals, a number of epidemiological studies evaluated the effects of children's exposure to these compounds. Children accidentally exposed to high levels of PCDD/Fs either before or after birth have been reported to present a number of developmental deficits (Charnley and Kimbrough, 2006). For that reason, in this work the level of PCDD/Fs in lichens overlayed to infant population (0-14) was also plotted (Figure 3). Overall, this environmental information may allow the assisted selection of populations with different potential risks of PCDD/Fs exposure for further health studies.

CONCLUSIONS

The construction of reliable atmospheric pollutant spatial models provides the location of the areas with greatest/lowest pollutant deposition, integrated over time. Those models would be very useful in Human Biomonitoring and/or epidemiological studies for selecting control and exposed groups of population. It would allow saving resources because gives the possibility to focus human biomonitoring and/or epidemiological studies in areas with effective pollution impact.

The regional maps developed in this study can be used to: i) identify critical PCDD/F deposition areas; ii) optimise PCDD/F monitoring networks; iii) produce risk assessment studies, including epidemiological investigations. The methodology followed to perform this study can be applied to other regions of the world, thereby contributing to a better knowledge of PCDD/F deposition levels in ecosystems and its impact on human health.

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4.2 | Assessing human exposure to PAHs in a petrochemical region based on data from environmental biomonitors

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ABSTRACT

Polycyclic aromatic hydrocarbons are toxic compounds which have been classified by the International Agency for Research on Cancer as probable or possible human carcinogens. Human exposure to PAHs is usually assessed considering data from a single air monitoring station as being representative of a large region; however, air pollution levels can change at small spatial scales and thus also the environmental exposure. The use of environmental biomonitors is a useful tool to assess the levels of PAH with high spatial resolution. The aim of this work was to assess human exposure to PAHs in a petrochemical region in Portugal, integrating data from environmental biomonitors (lichens), air and soil in a regional area; and to assess the health risk associated with the exposure to PAHs with high spatial resolution. For that, benzo[a]pyrene equivalent concentrations (BaP) in samples of soil, air and lichens collected in the study region were used to assess human exposure through different pathways: inhalation of air and soil particles, ingestion of soil and dermal contact with soil. Human health risk was calculated through the Incremental Lifetime Cancer Risk (ILCR). We found that BaP equivalent concentrations in the region ranged from 6.90 to 46.05 ng BaPeq/g in lichens, from 16.45 to 162.02 ng BaPeq/g in soils, and from 0.02 to 0.16 ng BaPeq/m^3 in air showing the high variability in a regional area. Human exposure to PAHs varied between 976 ngBaPeq/day and 42877 ngBaPeq/day. ILCR showed values between 10^{-4} and 10^{-3} considering all exposure pathways. Considering only inhalation, ILCR showed values between 10^{-6} and 10^{-5}. The main risk seemed to come from soil (either ingestion or inhalation of ressuspended soil particles). The high spatial resolution of our environmental data allowed detecting critical exposure levels at unexpected sites. Our results identified important areas where health studies on local populations should be focused, and where environmental levels of PAHs should be monitored over time in order to protect human health.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a large class of organic compounds which are products of incomplete combustion. They are also present in petroleum and coke products. They occur in the environment as complex mixtures of many components with varying toxic levels (Nisbet and LaGoy, 1992). The US Environmental Protection Agency (EPA) has promulgated 16 unsubstituted PAHs (EPA-PAH) as priority pollutants to be monitored in the environment. Several compounds of this group have been classified by the International Agency for Research on Cancer (IARC) as probable (2A) or possible (2B) human carcinogens (IARC, 1987). Human skin, lungs, and bladder cancer have been

associated with PAHs (Boffetta et al., 1997). Additionally, several individual PAHs, such as benzo[*a*]pyrene, chrysene, indeno[1,2,3-*c,d*]pyrene, and benzo[*b*]fluoranthene have produced carcinogenic, mutagenic, and genotoxic effects in animal experiments (Thyssen et al., 1981; Deutsch-Wenzel et al., 1983). Somers et al. (2002, 2004) have also found that air pollution enriched with PAHs induce heritable (paternal germ-line) mutations in mice. More recently, PAHs have been associated with elevated levels of DNA adducts (PAH-DNA adducts) and P53 mutations in persons who smoke or are exposed to PAH in the workplace and ambient air (Alexandrov et al., 2002; Gaspari et al., 2003). Perera et al. (2002) has also indicated that airborne PAHs have been implied in human reproductive effects, PAH-DNA adducts in newborns as well as preterm birth and intrauterine growth restriction.

Human exposure to PAHs can occur by various pathways, notably inhalation of air and resuspended soil particles, ingestion of food (which may include maternal milk), water and soil particles (relevant in the case of children), and dermal contact to soil and water (EPA, 1998). To assess human exposure, usually concentrations of PAHs deposited in air, soil, water, etc. are estimated based on emission data from known pollution sources; this is made in order to assess the impact of specific human activities, namely the industrial ones. However, the uncertainty associated with the places where pollutants are predicted to deposit and where they actually are being deposited is very high, increasing also the uncertainty of the exposure data. Environmental observations for regulatory purposes are performed at air quality monitoring stations, using high-volume samplers to capture PAHs retained in particulate- and vapor-phases of atmosphere. These stations, normally only few in space, tend to be located at specific sites selected for their expected relatively high or low concentrations and are often placed at a much higher altitude than the human breathing zone (EHC 27, 1983). Moreover, the measurements reflect a short-term indicator that varies in time and which does not reflect the levels that populations are exposed in space and in the long-term. In order to perform reliable epidemiological studies, environmental observations must be made in a way that reflects as closely as possible the exposure of the population under observation. One possible way to perform an accurate measurement of the environmental level of a particular pollutant is through a biomonitoring program in a spatial explicit scale (EHC 27, 1983). Environmental biomonitoring consists in the monitoring of the pollutant levels in the environment by means of living organisms

(Martin and Coughtrey, 1982; Puckett, 1988; Sloof, 1993). Within biomonitors, lichens (symbiosis between fungi and algae and/or cyanobacteria) are one of the most used organisms to monitor atmospheric deposition of several air pollutants (Branquinho, 2001). Lichens are long-lived biomonitors, and thus they are long term integrators of the atmospheric pollution deposition. This characteristic is of crucial importance for evaluating human exposure to pollutants such as PAHs; time integration of these compounds allows relating low levels of pollutants with long-term chronic effects on health (Augusto et al., 2007). Some authors have shown that lichens reflect atmospheric deposition of PAHs and thus can be used as estimators of atmospheric pollutants (Augusto et al., 2009, 2010; Guidotti et al., 2003; Blasco et al., 2006, 2007, 2008). In what concerns the environmental health studies, lichens allow us to obtain information about environmental levels of pollutants with a high spatial resolution, as they can be collected from a number of sites using low resources (or in a cost-effective way). The application of biomonitoring with lichens to human health studies was previously shown for PCDD/Fs (Augusto et al., 2007).

For the first time the assessment of human exposure to PAHs was based on the use of lichens as estimators of air concentrations, and thus estimators of what humans are exposed to through inhalation. The aim of this work is to assess human exposure to PAHs in the surroundings of a petrochemical industrial complex in a spatial explicit way, integrating data from environmental biomonitors (namely lichens as estimators of air PAH levels) and soil; and to assess the health risk associated with the exposure to PAHs with high spatial resolution.

EXPERIMENTAL SECTION

Study area

This study was developed in the highly industrialized region of Sines, located on the SW coast of continental Portugal, facing the Atlantic Ocean. This region encompasses several important industrial facilities established in the late 1970s: a coal-fired power station, an oil refinery, a chemical plant, and more recently an industrial landfill as well as many other smaller industrial plants, based primarily on the processing of oil products. Moreover, urban areas have recently increased. During the last decade awareness has increased among populations regarding the environmental and human health impact of

pollutants which may be emitted by industries in the region. Three main cities can be found in the area, namely Sines (with an area of 151 km^2 and 12461 inhabitants), Santiago do Cacém (with 120 km^2 and 7274 inhabitants), and Santo André (with 75 km^2 of area and 10696 inhabitants). We've focused our study in the closest four parishes to the industrial center, namely Sines (S), Santiago do Cacém (SC), Santo André (SA), and Santa Cruz (SZ).

Environmental monitoring data

In January 2008, during 3 days under constant climatic conditions (no rain, the same range of temperature, humidity, wind speed and direction), 34 lichen samples of the species *Parmotrema hypoleucinum* (Steiner) Hale were collected at a number of sites within the highly industrialized region of Sines. The sampling design followed a two kilometer grid that had previously been selected for different studies (Pinho et al., 2008). The lichen *P. hypoleucinum* was selected because it is ubiquitous and tolerates a variety of land-uses, such as urban, industrial, forestry, and also background areas. The collection was made mainly from branches and trunks of *Quercus suber* L. (cork-oak) (N=25), from *Pinus pinea* L. (umbrella-pine) (N=4), and some exceptions from other tree species (especially in urban areas). Samples were packed in brown glass bottles, protected from sunlight, and immediately stored at 4 °C. At 26 sites (24 matching the sites of the lichen sampling points), soil samples were also collected. Samples were taken from the upper 5 cm of soil and placed in polyethylene bags. Once in the laboratory, soil samples were sieved through a 2 mm mesh screen, transferred to glass bottles in order to prevent adsorption by plastic, protected from sunlight and stored at 4ºC. All samples were extracted and analyzed for the 16 EPA-PAHs within 2 months. Particle concentration ($\mu g/m^3$) was measured at two air quality monitoring stations of the National Air Quality Network, located at the parish S, one at an urban site and the other at an industrial site. The sampling was made from January to September 2008, using high-volume samplers and cellulose filters (retaining TSP and PM10). The mean particle concentration from both sites were used afterwards and assumed to be the same for all parishes.

PAH analyses

All PAH analyses took place at the certified laboratory of the Portuguese Environmental Protection Agency (APA). For lichen and soil analyses, approximately 2 g of sample was

extracted in a Soxhlet with 200 mL of acetonitrile for 24 h. After extraction, all extracts were concentrated by rotary vacuum evaporation and cleaned-up in a florisil column with 30 mL of acetonitrile as eluting solvent. Subsequently, the extracts were again evaporated and concentrated with a gentle stream of purified N2 to 1 mL. The samples were analyzed by a high-performance liquid chromatograph (Hewlett Packard), using two columns (Agilent C18 and Phenomenex C18), coupled to an ultraviolet/visible detector (DAD/V–UV) and to an ultraviolet fluorescence detector (FLD). The 16 EPA-PAHs were analyzed, namely: acenaphtylene, naphthalene, fluorene, phenanthrene, fluoranthene, chrysene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, acenaphtene, anthracene, pyrene, benzo[a]pyrene and indeno[1,2,3-cd]pyrene. Organic matter content of the soil samples was evaluated according to the Loss of Ignition (LOI) method. Samples were dried in order to eliminate water content. Subsequently, they were heated for 2 h at 600 ºC and the weight loss was assessed. The potential of soils for accumulating PAHs mainly depends on their organic matter content and the size of their particles; in this study, sand was the more frequent soil size fraction, with a mean organic matter content of 2.7%, varying from 0.3 to 12.3%, and with no significant correlation between this variable and the concentration of PAHs (data not shown).

BaP equivalent concentrations

The carcinogenic risk of a PAH mixture is often expressed by its benzo[a]pyrene equivalent concentration (BaPeq). Based on the carcinogenic potency of each other individual PAH relative to that of BaP (Toxic Equivalent Factors, TEFs), the carcinogenic potency of each PAH in the mixture is expressed by its BaPeq. There are different TEFs developed by different agencies and scientists (MOE, 1997; USEPA, 1993; CEPA, 1994; Cal EPA, 1993, Nisbet and LaGoy, 1992). In our study we've decided to adopt TEFs developed by Nisbet and LaGoy (1992), as these values are most commonly used while assessing the carcinogenic potency of PAH mixtures (Tsai et al., 2001). In this way, for each sample it was calculated the total carcinogenic potency through the sum of BaPeq concentrations calculated for each of the sixteen compounds.

Spatial analysis of environmental data

The BaP equivalent concentrations measured in lichen samples were translated into equivalents for air concentrations, using a calibration model published elsewhere

(Augusto et al., *submitted)*. Calibration, which took place in the same region as the one of this study, was performed as follows: over a nine-month period (January to September 2008), 143 568 m^3 of air was sampled and 90 different samples were collected. Each sample corresponded to a 24 h period of continuous sampling. The gap between sampling periods was 48 hours. Native lichens of the species *P. hypoleucinum* (Steiner) Hale were collected close to the high volume air sampler, every 15-days from February to May and every 30 days from June to September 2008 – a total of 13 lichen samples were collected. All samples were extracted and analyzed for the 16 EPA-PAHs; filters from each 15-days (from January to September) were polled together – a total of 19 sample groups was obtained. BaP equivalent concentrations in lichens and air varied from 2.67 to 16.96 ngBaPeq/g and 0.0040 to 0.1143 ngBaPeq/m^3, respectively, covering a considerable range of concentrations; for both, lichens and air, greatest concentrations were found during winter. Since there was a significant correlation (r=0.7975, p=0.002) between lichens and air we assumed that a calibration between both was possible. Translation of PAHs in lichens into the equivalent ones in air was made using the formula: Air ($ngBaP_{eq}/m^3$) = 0.003 × Lichen ($ngBaP_{eq}/g$) - 0.0043. The BaP equivalent concentrations translated into equivalents for air concentrations were assumed to be indicators of PAH levels in the air. For each set of data (estimated air and soil PAH concentrations) a spatial model was built. In a first step, spatial correlations between samples were generalized in a correlation function of distance between any two points, the semivariogram, which summarizes the main spatial continuity patterns of the attributes. For this purpose we fitted isotropic spherical models to the experimental semivariograms of the attributes. The variables exhibit similar spatial patterns with amplitudes around 3000 m. In a second step, a least-squares linear regression algorithm, the ordinary kriging, was applied to estimate grid maps for each attribute taking into account the models spatial dependence previously fitted. Then, using ESRI ArcMap 10.0 tools a vectorial layer of the *Carta Oficial Administrativa Portuguesa 2010* (*Administrative Portuguese Official Map*) from Instituto Geográfico Português (*Portuguese National Authority in Cartography*) was overlaid to the grid maps. Afterwards, the statistics of the estimated grid values for each variable were calculated inside each “parish” polygon for all variables.

Human exposure assessment

Human exposure to PAHs was calculated based on BaP equivalent concentrations (BaPeq) measured in environmental samples (estimated air and soils). This study was made analyzing the exposure of population living in each studied parish (S, SC, SA and SZ). No distinctions between genders were made. Populations were sub-grouped by age, namely infant (0-9 years old), children (10-19 years old), and adult (>19 years old). Demographic data at a parish level were obtained from the *Censos 2001* from *Instituto Nacional de Estatística* (*Portuguese National Authority in Statistics*). Human exposure estimates were calculated with the mean concentrations of PAHs in the study area for each parish. Using the high-end approach, the maximum concentrations were used.

Human exposure was considered for: i) inhalation of air; ii) inhalation of resuspended particles from soil; iii) ingestion of soil (important in infant and children exposure assessments); and iv) absorption by skin contact with soil (relevant in the case of children and also adults that work as farmers). Pathways through ingestion of food, water and also breast feeding, were not considered due to lack of data at parish level for the study area and we assumed these exposures were similar between parishes and thus not contributing significantly to the differences in the spatial exposure. Moreover, most of the foodstuffs are produced elsewhere and local environmental and health agencies do not have the power to control that production. The algorithms for each exposure pathway were adapted from Schumacher et al. (2001) and specific parameters for each age group were adapted from other references (ICRP, 1994; Nessel et al., 1995; US EPA, 1998; US EPA, 1990; Katsumata and Kastenberg, 1997). Algorithms and parameters used in the work are displayed in Table 1.

Human health risk assessment

In this study it was only considered the carcinogenic effects produced by these compounds in humans and not al the other effects that they might produce. Risk characterization combines exposure and dose-response assessments. For carcinogenic effects, risk is expressed as excess probability of contracting cancer over a lifetime – Increment Lifetime Cancer Risk (ILCR). The US EPA has developed cancer slope factors (CSFs) for carcinogenic effects. In the current study, inhalation, oral and dermal exposures were considered separately. CSFs values for inhalation (6.10 mg/kg/day) and oral (7.30 mg/kg/day) exposures were quoted from Smith (1996), while for dermal

exposure (25 mg/kg/day) quoted from Knafla et al. (2006). CSFs do not represent a safe exposure level, but relate the exposure to the probability of causing carcinogenic effects (Gold et al., 1995). The carcinogenic risk was calculated by multiplying the estimated dose by the cancer potency factor. A total pathway risk was calculated by summing the cancer risk estimated for each pathway. Since cancer risks describe the probability of developing cancer over a lifetime, the entire duration of exposure must be considered for risk assessment. In this study we assumed 70 years to be the average lifetime for the population of the area and that during this period individuals would be exposed to environmental PAHs.

TABLE 1. Algorithms and parameters used for calculation of daily individual exposure. Algorithms were adapted from Schumacher et al (2001).

Algorithms		Parameters		Age group		
				0-9	*10-19*	*>19*
Inhalation of air (Inhl)	Vr[1]	Ventilation rate	m^3/day	5.96	24.87	32.74
	AFinhl[2]	Absorption fraction for inhalation	unitless	100	100	100
Inhl = Ac * Vr * AFinhl	Sir[3]	Soil ingestion rate	mg/day	300	300	150
	Afings[2]	Absorption fraction for ingestion of soil	unitless	40	40	40
Ingestion of soil (Ings)	RET[2]	Fraction retained in lungs	unitless	60	60	60
	SA[3]	Exposed skin surface	m^2	0.7	1.5	1.9
Ings = Sc * Sir * AFings	CT[3]	Contact time soil-skin	hr/day	1.5	1.5	1.9
	AF[4]	Soil to skin adherence factor	mg/cm^2	1	1	1
Inhalation of ressuspended particles from soil (Inhlas)	AFd[5]	Absorption factor for dermal contact	unitless	0.003	0.003	0.003
Inhlas = Sc * RES * Vr * RET * Pa * AFinhl	Ac	Air concentration	ngTEQ/m^3			
	Sc	Soil concentration	ngTEQ/g			
Dermal absorption exposure (DAE)	Pa	Particle concentration	µg/m^3			
	RES	Ressuspended particles from soil	unitless			
DAE = Sc * SA * CT * AF * AFd	Inhl	Inhalation of air	ngTEQ/day			
	Ings	Ingestion of soil	ngTEQ/day			
	Inhlas	Inhalation of particles	ngTEQ/day			
	DAE	Dermal exposure	ngTEQ/day			

Adapted from: [1] ICRP (1994); [2] Nessel et al. (1995); [3] US EPA (1998); [4] US EPA (1990); [5] Katsumata and Kastenberg (1997)

RESULTS AND DISCUSSION

Environmental levels of PAHs

In this study we found that BaP equivalent concentrations measured in lichens ranged from 6.90 to 46.05 ng BaPeq/g, with a mean value of 12.12 ngBaPeq/g; while in soil ranged from 16.45 to 162.02 ng BaPeq /g with a mean value of 25.70 ng BaPeq/g (for details regarding concentrations of each PAH compound see Augusto et al., 2010). Estimated air concentrations based in lichens ranged from 0.02 to 0.16 ng BaPeq/m^3 with a mean value of 0.04 ng BaPeq/m^3. In the interpolated map of the air concentrations (Figure 1a) it is possible to distinguish the industrial areas (darkest

areas) and locate them in the study region. The darkest areas match the areas where concentrations of PAHs were the highest. On the other hand, in the interpolated map of the soil concentrations (Figure 1b), the darkest areas are more restricted to a specific industrial site, where a small village is located, and to the urban area of the parish SC. This specific industrial site is located close to a coal-fired power plant in the southern limit of the main industrial region. However, pollutants, once emitted from industries or other sources, tend to be transported south way, following the prevailing wind direction of this region, which is from NW. In this way, this specific area receives pollutants from the entire industrial region, as confirmed by the interpolated map of air that showed the southern part of the study area to be more contaminated than the northern one (Figure 1a). In addition, in the southern part of the study region the area covered by naked soil (without vegetation) is larger and the area occupied by forest is smaller than in the northern part; this means pollutants are less intercepted by tree canopies, which act as barrier in deposition to soil. Tree canopies have been shown to play an important role in the interception of pollutants (Bakker et al., 2000). Additionally, other studies performed in the same region have shown that the highest deposition of PAHs occurs in mixed urban and industrial areas, especially for the high molecular weight PAHs which are considered to be more dangerous to human health (Augusto et al., 2009).

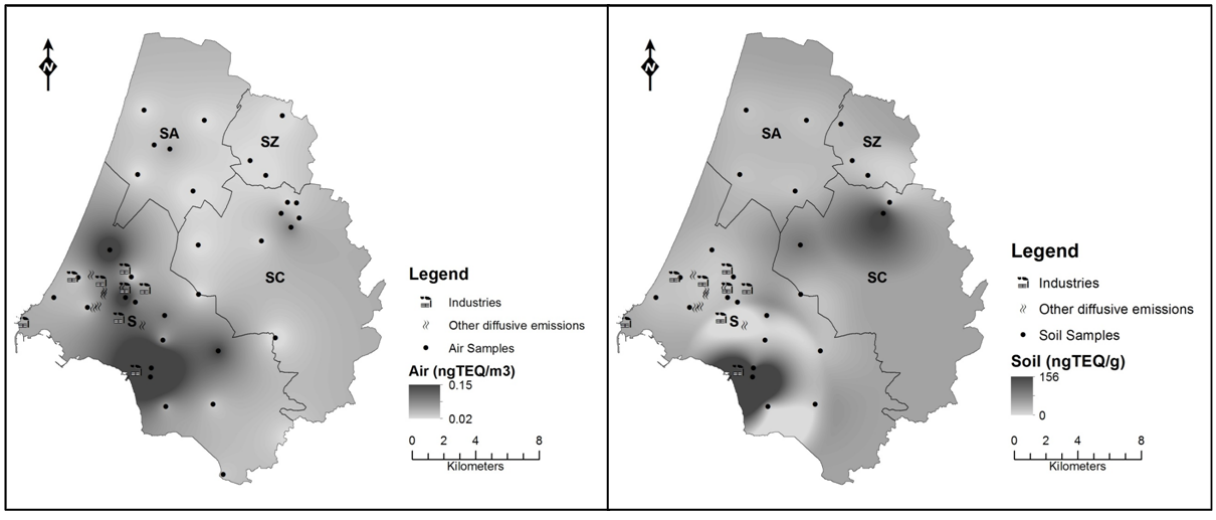

Figure 1. Maps of the interpolation of PAHs in air (measured in lichens and translated into air equivalent concentrations) (a) and in soil (b). TEQ correspond to BaP equivalent concentrations (BaPeq). Parishes: S (Sines), SA (Santo André), SZ (Santa Cruz) and SC (Santiago do Cacém).

Potential health impact based on risk-based concentrations

Smith (1996) has developed a method to assess the potential health impacts of a given PAH concentration measured in environmental samples. The author has combined toxicological constants with predetermined risk levels and protective human exposure assumptions to produce risk-based concentrations for 596 contaminants in air, soil, drinking water and edible fish (Smith, 1996). Following this method, we assessed the potential health impact through the ratio between BaP equivalent concentrations measured in soil and estimated for air, and the risk-based concentrations reported by Smith (1996) for BaP. We found a potential health impact in the parish S for the maximum value detected in soil samples (Table 2).

TABLE 2. Statistical summary for total BaP equivalent concentrations for air (estimated through lichens) and soil at a parish level. Potential health impact calculated through the ratio between measured concentrations and risk-based concentrations proposed by Smith, 1996. Potential health impact exists when the ratio is bigger than 1.0. Number of inhabitants at each parish is also displayed.

Parish		Environmental concentrations		Potential Health Impact (risk when >1)*			Inhabitants		
Parishes		Air (ng BaPeq/m3)	Soil (ng BaPeq/g)	Ambient Air	Industrial Soil	Residential soil	0 - 9	10 - 19	> 19
SZ	Mean	0.0283	18.88	0.028	0.024	0.215	25	41	434
	SD	0.0016	2.87						
	Max	0.0316	23.04	0.032	0.030	0.262			
SC	Mean	0.0327	24.99	0.033	0.032	0.284	587	771	5916
	SD	0.0026	5.20						
	Max	0.0463	49.66	0.046	0.064	0.564			
SA	Mean	0.0308	19.57	0.031	0.025	0.222	929	1565	8202
	SD	0.0026	1.96						
	Max	0.0474	27.49	0.047	0.035	0.312			
S	Mean	0.0428	23.20	0.043	0.030	0.264	1242	1541	9678
	SD	0.0132	15.67						
	Max	0.1524	156.12	0.152	0.200	1.774*			

Though this maximum correspond to the southern part of the industrial area, the existence of a small neighborhood with a few homes (≈20) makes us to consider this soil as being a mix of residential and industrial soil. In fact, one of the characteristics of several Mediterranean countries is that industrial areas are frequently overlapped to urban/residential areas, which makes the distinction between industrial and residential soils a difficult task (Augusto et al., 2004). The use of risk-based concentrations is a

general method to obtain a quick answer when prioritizing health problems; however it does not take into consideration the characteristics of specific populations. For this reason, it's always useful and more accurate to assess human exposure to pollutants and afterwards calculate the health risk for the exposed population.

Human exposure to PAHs and health risk

One of the aims of this work was to assess human exposure to environmental PAHs, in order to find which sub-populations in the study area were more exposed. For this purpose, we first calculated the mean and maximum PAH concentrations for each parish and assumed that populations inside each parish were exposed to the same level of environmental PAHs. This assumption is important in terms of environmental management, policy making process, and also human health studies, as parishes are the smallest governmental unit at the country. In this work human exposure was calculated based on BaP equivalent concentrations measured in different environmental samples as an attempt to capture the risk existing at the present scenario. Environmental concentrations of PAHs may suffer changes in future, increasing or decreasing, and thus modifying the estimated human exposure.

We found that inhabitants of the parish S, where most industries are located, showed the highest exposure levels to PAHs at the high-end approach (considering the maximum environmental levels measured in each parish), varying between 9222 to 41305 ng BaPeq/day (age groups 0-9 and >19, respectively) (Table 3). Regarding mean levels, the parish SC showed the highest values for PAH human exposure for all age-groups, ranging from 1481 to 6639 ng BaPeq/day (age groups 0-9 and >19, respectively). This result was unexpected, as it would be more likely to have higher exposure levels at the main industrial area of the region (parish S). However, the high spatial resolution of our environmental data allowed detecting higher exposure levels at a parish that wouldn't be found using the data from the air quality monitoring stations that presently exist in the study region.

Total daily PAH intake for humans were estimated by de Kok and van Maanen (2000) to vary between 25000 to 300000 ng/day, excluding those individuals who are also occupationally exposed. Total daily PAH intake for humans for the general American population was estimated by Santodonato et al. (1981) to vary from 200 to about 20000

ng/day. These values include exposure through food and water ingestion, which were not included in our study, and which account for most of human exposure to PAHs.

Table 4 summarizes the results of the incremental lifetime cancer risk (ILCR) for each parish and age group. According to US EPA, a one in a million chance of additional human cancer over a 70 year lifetime (ILCR = 10^{-6}) is the level of risk considered acceptable or inconsequential (Asante-Duah, 2002). An additional lifetime cancer risk of one in a thousand or greater (ILCR>10^{-3}) is considered serious.

We found ILCRs between 10^{-4} and 10^{-3} for the total of the studied exposure pathways. Considering the mean values of exposure, all parishes showed ILCRs of 10^{-4}; while considering the high-end approach (maximum values) the parish S showed values of 10^{-3} for all age groups and CS for the age group between 10 and 19 years old. Regarding only inhalation of air, ILCRs were between 10^{-6} and 10^{-5}, which are considered reasonable levels. The main risk seemed to come from soil (either ingestion or inhalation of soil) (Table 4), which was validated by the potential health impact calculated through the ratio between the environmental concentrations and risk-based concentrations (Table 2).

TABLE 3. Daily individual exposure to PAH (ng BaPeq/day) for each parish and each age group. Parishes: SZ (Santa Cruz), SC (Santiago do Cacém), SA (Santo André) and Sines (S).

		SZ		SC		SA		S	
Age group	Exposure pathway	Mean	Max	Mean	Max	Mean	Max	Mean	Max
0-9	Inhalation of air	14	15	18	28	16	22	30	96
	Ingestion of soil	197	197	396	598	198	198	335	1944
	Inhalation of soil particles	765	765	1534	2315	766	768	1299	7532
	Dermal exposure	0.04	0.04	0.09	0.13	0.04	0.04	0.07	0.43
Daily individual total exposure (ngBaPeq/day)		***976***	***977***	***1948***	***2941***	***980***	***988***	***1664***	***9573***
10-19	Inhalation of air	58	61	76	118	67	91	126	402
	Ingestion of soil	197	197	396	598	198	198	335	1944
	Inhalation of soil particles	3191	3191	6400	9659	3197	3204	5419	31429
	Dermal exposure	0.09	0.09	0.19	0.28	0.09	0.09	0.16	0.91
Daily individual total exposure (ngBaPeq/day)		***3446***	***3450***	***6872***	***10375***	***3461***	***3494***	***5881***	***33776***
>19	Inhalation of air	76	81	100	156	88	120	166	529
	Ingestion of soil	99	99	198	299	99	99	168	972
	Inhalation of soil particles	4200	4200	8426	12716	4208	4218	7134	41375
	Dermal exposure	0.06	0.06	0.12	0.18	0.06	0.06	0.10	0.58
Daily individual total exposure (ngBaPeq/day)		***4375***	***4380***	***8723***	***13171***	***4395***	***4437***	***7468***	***42877***

TABLE 4. Incremental lifetime cancer risk (ILCR) for each parish and considering each exposure pathway and age-group.

Incremental lifetime cancer risk (ILCR)	SZ		SC		SA		S	
	Mean	Max	Mean	Max	Mean	Max	Mean	Max
Inhalation of air	2,4E-06	2,6E-06	3,2E-06	4,9E-06	2,8E-06	3,8E-06	5,3E-06	1,7E-05
Ingestion of soil	4,1E-05	4,1E-05	8,3E-05	1,2E-04	4,1E-05	4,1E-05	7,0E-05	4,1E-04
Inhalation of soil particles	1,3E-04	1,3E-04	2,7E-04	4,0E-04	1,3E-04	1,3E-04	2,3E-04	1,3E-03
Dermal exposure	3,1E-08	3,1E-08	6,2E-08	9,3E-08	3,1E-08	3,1E-08	5,2E-08	3,0E-07
ILCR (0-9)	**1,8E-04**	**1,8E-04**	**3,5E-04**	**5,3E-04**	**1,8E-04**	**1,8E-04**	**3,0E-04**	**1,7E-03**
Number of subjects developing cancer in their lifetime	*0,0044*		*0,2073*		*0,165*		*0,3746*	
Inhalation of air	5,5E-06	5,8E-06	7,2E-06	1,1E-05	6,3E-06	8,7E-06	1,2E-05	3,8E-05
Ingestion of soil	2,2E-05	2,2E-05	4,5E-05	6,8E-05	2,2E-05	2,3E-05	3,8E-05	2,2E-04
Inhalation of soil particles	3,0E-04	3,0E-04	6,1E-04	9,2E-04	3,0E-04	3,0E-04	5,2E-04	3,0E-03
Dermal exposure	3,6E-08	3,6E-08	7,2E-08	1,1E-07	3,6E-08	3,6E-08	6,1E-08	3,6E-07
ILCR (10-19)	**3,3E-04**	**3,3E-04**	**6,6E-04**	**1,0E-03**	**3,3E-04**	**3,4E-04**	**5,7E-04**	**3,2E-03**
Number of subjects developing cancer in their lifetime	*0,0136*		0,5094		0,5208		0,8712	
Inhalation of air	4,7E-06	5,0E-06	6,2E-06	9,7E-06	5,5E-06	7,5E-06	1,0E-05	3,3E-05
Ingestion of soil	7,4E-06	7,4E-06	1,5E-05	2,2E-05	7,4E-06	7,4E-06	1,2E-05	7,2E-05
Inhalation of soil particles	2,6E-04	2,6E-04	5,2E-04	7,9E-04	2,6E-04	2,6E-04	4,4E-04	2,6E-03
Dermal exposure	1,5E-08	1,5E-08	3,0E-08	4,5E-08	1,5E-08	1,5E-08	2,5E-08	1,5E-07
ILCR (>19)	**2,7E-04**	**2,7E-04**	**5,5E-04**	**8,2E-04**	**2,7E-04**	**2,8E-04**	**4,7E-04**	**2,7E-03**
Number of subjects developing cancer in their lifetime	*0,1187*		*3,2268*		*2,2539*		*4,5186*	

Cancer risk assessment of individual PAHs and PAH mixtures are based mainly on laboratory tests performed in animals and occupational epidemiological studies (Bostrom et al., 2002). Moreover, most studies consider only one exposure pathway, such as inhalation of contaminated air, when assessing cancer risk. Risk estimation of PAH exposures is a complex issue. PAHs in the environment comprise several hundred compounds, most of which occur together with a large number of other carcinogenic pollutants. In addition, people are exposed to other sources of PAHs other than environmental, such us tobacco smoking, certain occupational exposures (coke production industries, coal gasification, aluminum production, etc.), food and water ingestion; all these increases the uncertainty in cancer risk assessment.

In this study, we aimed to assess human exposure to environmental PAH considering a set of exposure pathways, based on environmental data with high spatial resolution. This kind of approach is innovative as it was made for general population (not for occupational exposed population) and it allowed identifying critical areas where health studies on local populations should be focused; and where environmental levels of PAHs should be monitored over time in order to protect human health.

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Chapter 05 |

General discussion

Chapter 05 | General discussion

5.1. Introduction

The ultimate aim of this thesis was to develop a technology for biomonitoring persistent organic pollutants (POPs) and for evaluating their impact on ecosystem and human health. As lichens have been the most used biomonitors in terrestrial environments to achieve pollution, in this thesis the main focus was given to these organisms. Though many studies have been published using lichens as biomonitors of a wide range of elements, regarding POPs only a few studies were performed, and thus the use of lichens as POP biomonitors was still in a germinal stage and needed further study. During the thesis emphasis was given to POPs that are not intentionally produced – like PCDD/Fs and PAHs.

In this sense, in order to optimize the use of lichens as POP biomonitors, a first set of studies was carried out in this thesis, regarding the **factors that contribute for the interception and accumulation of PCDD/Fs and PAHs in lichens**. Questions regarding the influence of the growth form of the lichen, its age and composition, as well as the influence of the substrate they're collected from, were analysed. All these factors have shown to be sources of variability when using lichens for biomonitoring other elements, such as heavy metals, and thus there was a need to understand how they affect lichen performance as POP biomonitors. In the same way, **climatic factors**, such as dry/wet deposition and temperature, have been pointed out as important factors influencing POP deposition and also lichen interception and accumulation, and thus were also studied in this thesis.

In a second set of studies there was a need to understand **what are lichens reflecting – soil or air?** Can lichens be used to evaluate atmospheric deposition of POPs or are, in the contrary or in addition, reflecting POP soil ressuspension? POP concentrations and profiles in lichens were compared to the ones found for air, soil and also for pine needles (one widely used biomonitor of POPs). In this set of studies, it was also priority to establish a calibration between lichens and air and soil, so that lichens could be included in regulatory monitoring schemes.

One of the major challenges in environmental monitoring studies is to **track pollution sources**. This can be a tricky task in multisource environments, where different kinds of

industries, urban activities, agricultural practices, etc., are all contributing to the input of POPs in the environment. A desirable biomonitor technology would be the one capable of making this distinction, and thus in this thesis it was studied the viability of using lichens to accomplish this aim. As lichens can't be found in aquatic environments, another biomonitor – an aquatic bryophyte – was used to assess POP pollution in water streams.

One of the final applications of the technology developed in this thesis, and which is the most important, was its use in **human health studies**. In environmental health studies, when the aim is to relate pollution to human health, one of the major limitations is to assess which populations should be considered as control and which ones should be considered exposed. This limitation is a consequence of the lack of spatial resolution of pollutant deposition data. Usually, data from a single air quality monitoring station is considered as representative of a large area, and thus the level of human exposure to pollutants is considered to be the same all over the region. In this thesis, environmental biomonitors (lichens) were used to obtain pollution data with high spatial resolution and were used as long-term accumulators providing information regarding human chronic exposure to POPs through inhalation.

Finally, the integration of the information obtained in this thesis allowed designing a **proposal of guidelines** to be used when using environmental biomonitors to assess POP pollution.

5.2. Factors contributing for the interception and accumulation of POPs in lichens

Different lichen species have been used during the last years to monitor atmospheric deposition of POPs, but none of these studies have addressed the factors that contribute for the interception and accumulation of POPs by these organisms neither the variability between different lichen species – inter-specific variability (Augusto et al., 2004; Guidotti et al., 2003; Domeño et al., 2006; Blasco et al., 2006-2008, Shukla and Upreti, 2009). Among the factors that have been described to influence interception and accumulation of pollutants by lichens, it can be highlighted the growth form, lichen surface features, lichen age, and substrate where lichens are collected from.

Regarding the **growth form**, in this thesis it was shown (in chapter 2.1) that fruticose lichens accumulate greater concentrations of PCDD/Fs than foliose ones. It is well

known that lichen's morphology influences the rate at which lichens accumulate elements from the atmosphere (Garty, 2001). Growth form dictates thallus orientation and the amount of continuous surface area exposed to airborne deposition; therefore, it has a direct impact on the interception of atmospheric elements by lichens. Fruticose lichens have a bushy-like structure, with a higher surface/volume ratio than foliose lichens. This feature might facilitate the interception of aerosols and low molecular weight particles by fruticose lichens. McCrady (1994) when comparing four plant species, showed that the variability in the uptake rate constants for the compound 2378-TCDD (the most toxic PCDD/Fs) was reduced from a factor of 50 to 4 when the constants were normalised to the surface area of the plant. Similarly, Bohme et al (1999) found a significant relationship between uptake and surface area to volume ratios of different plants for gaseous compounds. Foliose lichens (with a flat, leaf-like structure, with well defined upper and lower surfaces) are less exposed to atmospheric deposition of POPs, given that only the upper side is exposed to the air.

Besides the surface/volume ratio, **lichen's surface** may also contribute to the retention of lipophilic compounds. Features such as roughness and gelatinous surfaces may facilitate the interception, uptake and retention of POPs both bound to particles or in the gas-phase. A high surface roughness leads to a higher turbulence and therefore to a higher supply of POPs (Branquinho, 2001).

When measuring POPs in plants (and lichens) some authors display results normalized to the extractable **lipid content** (Simonich and Hites, 1994; McCrady, 1994; Böhme et al., 1999). However, the extractable lipid content may not represent the actual storage volume, as POPs may also accumulate in non-extractable lipid material and/or POPs may not have reached internal lipids, which are included in the total lipid content (Simonich and Hites, 1994; McCrady, 1994; Böhme et al., 1999). In the case of metals, when these pollutants are deposited onto lichen surface, they can be retained by particulate entrapment, physicochemical processes such as ion exchange, and by passive and active intracellular uptake (Tyler, 1989; Branquinho, 2001). The mechanisms for POP uptake are likely to be similar, however as they're not soluble in water and they're highly lipophilic, they will probably bind to lipids, either in the surface of the lichen and inside the thallus.

Regarding the **inter-specific variability**, in this thesis different lichen species were analysed for POPs. In chapter 2.1 the fruticose lichens *R. canariensis* and *Ramalina fastigiata* (Pers.) Ach. and the foliose lichens *X. parietina*, *Parmelia caperata* (L.) Hale and *Parmotrema reticulatum* (Taylor) M. Choisy, collected at the same sites, were analysed for PCDD/Fs; and it was found that PCDD/F homologue profiles (contribution of each homologue group to the total PCDD/F concentration) were similar for most species, except for the foliose lichen *X. parietina*. In this foliose lichen, profile was dominated by the most chlorinated PCDD/Fs, such as OCDD/Fs and HpCDD/Fs, whereas in other lichen species, profile was dominated by the less chlorinated PCDD/Fs. The strongest contribution of the most chlorinated PCDD/Fs in *X. parietina* can be related to the **high longevity** of this lichen species. It has been suggested, when comparing lichens with pine needles in Norway, that the greatest POP concentrations found in lichens could be due to differences in plant ages (2 years for the pine needles and >5 years for lichens) (Ockenden et al., 1998). Another explanation for the strongest contribution of the most chlorinated PCDD/Fs in *X. parietina* can be related to the higher proportion of interception of ressuspended soil particles in relation to other lichen species (Guevara et al., 1995). It was also shown in chapter 2.1 that the most chlorinated PCDD/Fs in *X. parietina* are related to soil metals, such as Al and Fe (Augusto et al., 2009). Some authors, and in accordance with what was found in chapters 2.1 and 2.2, argue that the most chlorinated PCDD/Fs are more stable in the environment than the less chlorinated, and thus soil, acting as a sink, show greater concentrations of the most chlorinated PCDD/Fs (Domingo et al. 2001a,b; Augusto et al., 2010). The greatest contribution of the most chlorinated PCDD/Fs in *X. parietina* may subsequently have origin in soil ressuspension.

In biomonitoring studies requiring a high density of sampling sites and a regional-scale cover, where a high diversity of land uses may occur, such as industrial, urban and forestry, it's difficult to find the same lichen species over the whole territory. In such cases, the option is to use two or more lichen species to avoid gaps in the sampling grid. However, as different lichen species intercept and accumulate pollutants in different ways, an intercalibration between species is mandatory if we want to use more than one in the same survey. An example of this kind of **calibration** was reported in chapter 2.1, where it was found a positive linear correlation between concentrations of PCDD/Fs in the lichens *Ramalina canariensis* Steiner and *Xanthoria parietina* (L.) Th. Fr., even

though profiles were not similar. Calibration between these species is many times required, as they occupy different ecological conditions. While *R. canariensis* is tolerant to sea salt spray and thus is more frequent close to coastal areas, *X. parietina* is moderately tolerant to pollution and thus it can be easily found in urban areas where other lichens can't survive.

Regarding PAHs, in chapter 2.3 the foliose lichen *Parmotrema hypoleucinum* Steiner (Hale) was used as biomonitor and it was found that PAH profile was dominated by the 2-, 3- and 4-ring PAHs, the lowest molecular weight PAHs. The same was found in different studies for other lichen species, such as *X. parietina*, *Evernia prunastri* (L.) Ach. and *Parmelia sulcata* Taylor, whose profile was dominated mainly for the 3-ring PAHs (Blasco et al. 2006, 2007, 2008; Migaszewski et al. 2002; Guidotti et al. 2003; Domeño et al. 2006).

Substrates (phorophytes) where lichens are collected from may influence pollutant accumulation (Sloof and Wolterbeek, 1993). Lichens may uptake elements directly from substrate and/or through indirect atmospheric input: elements may be taken up from the atmosphere by the bark surface, followed by uptake into the lichen through the rhizines and/or after weathering of the bark surface (Sloof and Wolterbeek, 1993). This is particularly true for metals; but it hasn't been shown for POPs. Uptake of POPs into the lichen through the rhizines is not likely to happen, as POPs are hydrophobic compounds. It was shown in chapter 2.1 that lichens collected from different phorophytes, namely trees with different kind of canopies (*Olea europaea*, *Quercus suber*, *Quercus faginea*, *Pinus pinea*), have shown similar patterns of accumulation of PCDD/Fs. However, *X. parietina* collected from horizontal house roof-tiles has shown greater PCDD/F concentrations than those collected from horizontal trunks of Olive tree (chapter 2.1). The most likely is that canopies, independently of their shape, act as barrier for pollutants. In the case of POPs, Schönbuchner at el. (2001) showed that heavier POPs, which have greatest deposition rates, are easily intercepted by canopies, whereas lightest POPs remain longer in the atmosphere.

With this thesis it was found that fruticose lichens tend to accumulate greater concentrations of POPs than foliose ones, probably due to the highest surface/volume ratio. When comparing POP profiles, it was found that most species show similar profiles, with high contributions of lightest compounds (low molecular PAHs and less chlorinated

PCDD/Fs), except the foliose lichen X. parietina*, with a high longevity, which has shown an influence of POP soil ressuspension. Even though POP profiles between* X. parietina *and* R. canariensis *were different, it was possible to establish a calibration between POP concentrations, allowing using both species in the same biomonitoring study if necessary. Substrate has shown to not be a critical factor influencing POP interception and accumulation by lichens.*

5.3. Climatic factors

5.3.1. Atmospheric POP dry/wet deposition

Atmospheric POPs can be deposited to lichens by (dry) gaseous and (wet and dry) particle-bound deposition. Since solubility of hydrophobic POPs is very low in rain droplets or other precipitation, wet deposition of gases is of minor importance (Duinker and Bouchertall 1989; McLachlan and Horstmann 1998). Uptake of compounds that are volatilized from highly contaminated soil is another pathway (Trapp and Matthies 1997), particularly in lichens that are at the bottom part of phorophytes. Also, contaminated soil particles can be transported directly to the lichen surface by wind or splash (Jones and Duarte-Davidson 1997; Trapp and Matthies 1997). Once deposited on the surface of the lichen, compounds can be transported to other compartments of the lichen. Unlike plants, lichens don't have a cuticular wax layer, which controls and/or limits the transport of pollutants and thus POPs will diffuse through lichen surface. It has been demonstrated that high molecular weight POPs diffuse slower through plant surface than the low molecular weight ones (Schreiber and Schönherr, 1993; Bauer and Schönherr, 1992; Baur et al., 1996, 1997). High molecular weight POPs are most likely to be associated with particles and to remain at the surface of the lichen.

POP concentrations in lichens are influenced by dry and wet depositions. In fact, concentrations of PCDD/Fs in lichens tend to decrease after a wet deposition period. This decrease is greatest for the highest molecular weight compounds (the most chlorinated PCDD/Fs, such as OCDD), meaning that these compounds are probably associated with the surface of lichens (Figure 1). This association may be mainly due to a slower diffusion rate of the highest molecular weight compounds through the lichen thallus; the slower rate will cause a higher concentration of these compounds at lichen surface and thus will make them more susceptible to mechanical wash off. As POPs are

not soluble in water, rain is not likely to act as vehicle to lead POPs from the surface to the inside of lichens. Also, after a given volume of rain, the levels of PCDD/Fs in lichens remain relatively constant, meaning that a fraction of POPs will be captured inside the lichen thallus, or associated with insoluble particles trapped by the fungus, and not accessible to further wash off (Figure 2).

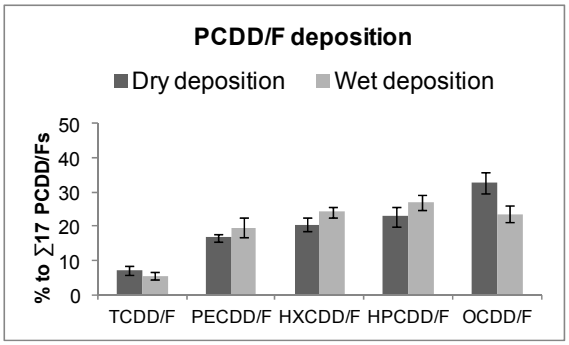

Figure 1. Homologue profiles for the 17 toxic PCDD/Fs for the fruticose lichen *R. canariensis* (n=4) collected from stone pines after a dry and a wet deposition period. Sampling was performed in the urban/industrial area of Setúbal peninsula, Portugal. Bars represent standard deviations. Concentrations of the 17 PCDD/Fs varied from 142.63±31.54 ng/Kg after the dry deposition period, to 77.30±15.99 ng/Kg after the wet deposition period.

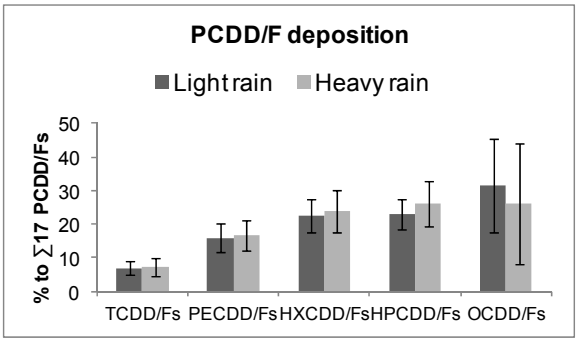

Figure 2. Homologue profiles for the 17 toxic PCDD/Fs for the fruticose lichen *R. canariensis* (n=20) collected after two distinct wet periods: after a long period of light rain (≈3 months) and after a heavy rain event after a long dry period. Sampling was performed in the urban/industrial area of Setúbal peninsula, Portugal. Concentrations of the Σ17 PCDD/Fs varied from 81.56 ±36.6 ng/kg after the light rain to 70.27±41.7 ng/kg after the heavy rain period.

5.3.2. Temperature

Regarding temperature, it has been stated that POP concentrations in air are greatest during winter (with cold temperatures) and lowest in summer (with high temperatures). As the ambient temperature decreases, partitioning to vegetation increases (Kömp and McLachlan, 1997). In this thesis it has been confirmed in chapter 2.4 a positive relationship between PAHs in atmosphere and in lichens. The same has been found in a few field studies, in which PAHs and PCDD/Fs were determined in the atmosphere and in vegetation in different seasons (Simonich and Hites, 1994; Nakajima et al., 1995; Wagrowshi and Hites, 1998). Vegetation and lichens react to temperature changes fast enough to observe a seasonal trend. In chapter 2.4, it was found significant negative correlations between PAH concentrations in lichens and temperature for PAHs. As temperature increases, PAH concentrations in lichens decrease. At highest temperatures and highest sunlight intensity, occurring during summer in European countries, there might exist a strongest evaporation and degradation of POPs, as well as chemical and photochemical reactions with ozone or OH radicals (Beyer et al., 2003; Schauer et al., 2003; Jung et al., 2010).

With this thesis it was shown that concentrations of PCDD/Fs in lichens tend to decrease after a wet deposition period, being this decrease greatest for the highest molecular weight compounds (the most chlorinated PCDD/Fs, such as OCDD), meaning that probably these compounds are mostly retained at lichens' surface. Regarding temperature, it was shown that as temperature increases, PAH concentrations in lichens decrease, which is in accordance with what happens with PAHs in air.

5.4. What are lichens reflecting - air or soil?

What are lichens reflecting –air or soil - is a major issue in biomonitoring studies. The perfect biomonitoring tool should reflect atmospheric POP, so that it could fulfil the gaps of the conventional monitoring methods (see chapter 1). In this thesis it was shown in chapters 2.3 and 2.4 that lichens are reflecting POPs existent in the air, either in the vapour- and particulate-phase. This same conclusion was achieved recently by Shukla and Upreti in India (2012). Temporal variation of the levels of PAHs in air (in the particulate-phase) and in lichens has shown the same trend; as concentrations of PAHs in air decrease, concentrations in lichens also decrease (chapter 2.4). Moreover, PAH

and PCDD/F profiles in lichens have shown to be similar to the ones found for air and much different from the ones found for soil (chapters 2.2 and 2.3). This similarity allows performing calibrations between concentrations of PAHs measured in lichens and in air (namely in the particulate-phase), enabling to translate concentrations in lichens into equivalent concentrations for air, and thus into regulatory values (chapter 2.4). For that, as lichens accumulate pollutants over time and have a "memory", it's essential to first assess which retrospective period are lichens reflecting. In chapter 2.4, when measuring PAHs in the lichen *P. hypoleucinum* and in air over time, it was shown that this species was reflecting mostly the PAHs measured in air during the previous 45 days; and thus this was the time span used to perform calibrations between lichens and air. Calibrations were performed for benzo-a-pyrene, which is considered an indicator of the overall mixture of PAHs, for the sum of the 16 priority EPA-PAHs and for its toxic concentration (BaP equivalent concentration of the Σ16 EPA-PAHs) (chapter 2.4). This kind of calibration is useful in regional studies, where air monitoring stations measuring POPs are in short supply, as it allows using data from biomonitors to match up to regulatory values. Regarding soil, it was shown in chapter 2.3 that even though PAH profiles in lichens and soil were not correlated, it was possible to establish a calibration between both for the Σ16 EPA-PAH concentrations, especially for the greatest values.

In this thesis it was shown that lichens are reflecting POPs existent in the air, either in the vapour- and particulate-phase. A calibration between lichens and air was possible, enabling to translate concentrations in lichens into equivalent concentrations for air, and thus into regulatory values. It was also shown that a calibration between lichens and soil is possible for the greatest concentrations.

5.5. Tracking sources of POPs using biomonitoring tools: natural and transplanted material

One of the main challenges in environmental monitoring studies is to be able to distinguish between different pollution sources. Deposition models are usually built (estimated) based on data from known emission sources (industries), not accounting with a set of unknown sources, such as small industries, urban and agricultural activities, which are also contributing to the input of pollutants into the environment, but are most of the times neglected. In addition, when POPs are released into

atmosphere they are subjected to a wide range of interferences, like temperature and chemical and photochemical reactions, wet and dry depositions, which will contribute to differences between what is emitted and what is really being deposited. Also, after being released in air or water, POPs tend to become diluted and thus concentrations measured at air monitoring quality stations (which measure pollutants for short periods of time) or in water samples show values under detection limits. Moreover, air quality monitoring stations are not enough to cover large territories; a single station (if existent) is usually considered representative of a whole region. All these constraints make source identification and allocation of POPs a difficult task. The use of biomonitors, such as lichens in terrestrial environments and aquatic mosses in aquatic environments (as shown in chapter 3.2), is a promising tool to track different sources of POPs. Besides the fact that these living organisms accumulate detectable concentrations of POPs inside their tissues, they can be easily collected from or transplanted to a high number of sites enabling to set a sampling grid dense enough to cover large territories (see next topic for further information regarding transplants). This kind of sampling grid will allow encompassing all the possible sources of POPs – known and unknown.

Biomonitoring tools have been used by several authors during the last years. Using the lichen *X. parietina* as PCDD/F biomonitor in an industrial and densely populated region of Portugal- Setúbal, it was possible to show that urban areas are responsible for a considerable input of pollutants in the environment (Augusto et al., 2004). In fact, both urban and industrial areas seem to be major focus of emission of PCDD/Fs (Augusto et al., 2004). This happens frequently in multisource areas, where industries overlap urban areas. During many years, it was attributable to industries the contamination of the environment, but the truth is that industries are nowadays subjected to tight regulations, whereas urban activities are still underestimated. In chapter 3.1, the lichen *P. hypoleucinum* was used to monitor the spatial deposition of PAHs in an industrial region of Portugal – Sines; and it was found that concentrations of PAHs were greater in mixed areas (areas with industries close to urban areas) when compared to single industrial or urban areas. In addition, when analysing PAH profiles, it was possible to distinguish between urban, industrial, forestry and agriculture; and within industrial, it was possible to distinguish between pyrogenic sources (with a higher contribution of 5- and 6-ring PAHs) and petrogenic sources (with a higher contribution of 2-ring PAHs) of PAHs. PAH profiles in urban areas were dominated by 4-ring PAHs, in forestry areas by

3-ring PAHs. Agriculture areas were not related to any specific PAH profile (see chapter 3.1).

A distinction between urban and industrial PAHs was also achieved by Shukla et al. (2011) using the foliose lichen *Pyxine subcinerea* Stirton in the south-western part of Uttarakhand state of India (in the foothills of Himalayas); these authors showed that lichens collected at industrial areas were enriched in 2-, 5- and 6-ring PAHs, whereas the ones collected at urban areas were enriched in 4-ring PAHs.

In aquatic environments, transplants of the aquatic moss *F. antipyretica* exposed for 3 months in water streams in a highly urbanized and industrial region of Portugal - Oeiras, showed that areas occupied by activities of tertiary and industrial sectors were responsible for a highest input of PAHs in the streams (mainly 2-, 3- and 5-ring PAHs and Σ16EPA-PAHs) if located less than 500 m and 1000 m, respectively; these PAHs were associated with enhanced Zn and Cu, and thus associated with a high traffic density (chapter 3.2).

When designing a sampling grid to monitor POPs, it's important to decide the sampling grid. For heavy metals, it's advisable to collect biomonitor samples every 100 to 500 meters, as these pollutants tend to deposit close to their emission sources. In the case of PCDD/Fs and PAHs, it was shown that their deposition ranges are larger than for metals; and ranges are strictly dependent on the size of the compound. For PCDD/Fs (Σ17 toxic PCDD/Fs), ranges between 9000 m and 16000 m were found when using the lichens *X. parietina* and *R. canariensis*, respectively (Pereira et al., 2004). The difference between species is mainly due to the fact that PCDD/Fs in *X. parietina* is mostly associated with particulate matter and thus will deposit close to emission sources. For PAHs, ranges between 2500 m (for 6-ring PAHs) and 10000 m (for 2-ring PAHs) were found in chapter 3.1, when using the lichen *P. hypoleucinum*. High molecular weight PAHs, which may have a pyrogenic industrial origin and be associated with particles, tend to deposit close to emission sources; on the other hand, low molecular weight PAHs, which may have a petrogenic origin, tend to be ubiquitous pollutants with a long-range transport power (Meharg et al., 1998; Nadal et al., 2009).

Ratios between PAH compounds and additional analysis of heavy metals have been used to track pollution sources using lichens (Shukla et al., 2011; Fernández et al. 2011). According to Yunker et al. (2002), anthracene/(anthracene+phenanthrene) ratio having

a value < 0.10 usually is an indicator of petroleum, while a ratio > 0.10 indicates dominance of combustion. Using this ratio, Shukla et al. (2011), in India, have found that combustion was the dominant source at the majority of industrial and urban monitored sites of the south-western part of Uttarakhand state, while at periurban sites PAHs were of petrogenic origin. This conclusion was supported by the use of an additional diagnostic ratio - total combustion PAHs/total PAHs (Hwang et al., 2003), with a value of 0.7, which revealed the predominance of combustion PAHs within the city and close to industrial sites. Using the ratio fluoranthene/(fluoranthene+pyrene), the same authors have found ratios > 0.5 within the city, which are characteristic of grass, wood, or coal combustion, and thus indicative of sources other than vehicular activity, wood and coal used for cooking purpose (Shukla et al., 2011; Yunker et al., 2002). The ratios fluoranthene/pyrene and phenanthrene/anthracene have been used by different authors as an indicator of PAH sources (Blasco et al., 2008; Fernández et al. 2011). A fluoranthene/pyrene ratio < 1 and phenanthrene/anthracene ratio < 10 indicate that pollution by PAHs is due to vehicular emissions and human (both industrial and domestic) activities with strong pyrolitic input (light and heavy PAHs can originate from the combustion of gasoline and diesel, respectively) (Shukla and Upreti, 2009). A phenanthrene/anthracene ratio > 10 is characteristic of petrogenic PAH pollution. Fernández et al. 2011, using these ratios, suggested that the major sources of PAHs in Caracas, Venezuela, were anthropogenic, petrogenic and mainly pyrogenic.

In chapters 3.1 and 3.2, crossing data regarding PCDD/Fs and PAHs with heavy metals, it was possible to disclosure different pollutant sources. Elements, such as Al and Fe are considered indicators of particulate matter, and thus a positive correlation between these elements and POPs (especially high molecular weight compounds) may indicate ressuspension of soil particles (chapter 3.1). A positive correlation between POPs and elements like Zn and Cr may indicate traffic and/or industrial origin; Cr may occur in refining operations and burning of residual oils (Nadal et al., 2009) and, when in addition to Zn, may indicate coal combustion in power generating plants (Huang et al., 2004). An association between POPs and Zn and Cu are indicative of high traffic density (chapter 3.2) (Augusto et al., 2004).

With this work it was demonstrated the viability of using biomonitors, lichens in terrestrial environments and bryophytes in aquatic environments, to track POP pollution sources. For

that, the analysis of POP profiles, land-use, and other pollutants (such as heavy metals) has shown to play an important role. The range of dispersion and deposition of a compound has shown to be dependent on its size. High molecular weight compounds tend to deposit close to emission sources, whereas low molecular weight ones tend to be ubiquitous and long-range transported.

5.6. Transplanted material

The use of biomonitor transplants (collection of biomonitors from a non polluted site and transplantation to sites to be monitored) is a useful tool either in terrestrial and aquatic environments. It's especially helpful when biomonitors are inexistent (or insufficient) in the sites to be monitored or when the aim is to assess pollutant deposition and accumulation rates. The drawback of this method is the quantity of biomonitors that should be collected at the non polluted site, particularly when the number of sites where transplants are to be exposed is high. This method has been widely used to monitor metal pollution in terrestrial and aquatic environments (Garty, 2001; Roy et al, 1996; Kelly et al., 1987); in the case of POPs, besides this thesis (chapter 3.2), there are a few publications regarding the use of transplants of aquatic mosses to monitor PAHs in water streams (Roy et al., 1996). In highly contaminated waters (close to harbours), PAH concentrations in aquatic mosses have shown to be strongly correlated with concentrations in water (Roy et al., 1996). However, in water streams most of the times POP concentrations in water samples show values under detection limits. Even if there are punctual POP discharges into the streams, POPs will become diluted in the water, will deposit and bioaccumulate in the living organisms. For this reason, the present regulatory methods to monitor POPs in water fail when attempting to disclosure pollutant sources. The use of aquatic moss transplants allows overcoming this limitation. In chapter 3.2, when using transplants of the aquatic moss *Fontinalis antipyretica* Hedw. to monitor PAH in urban and industrial water streams, it was shown that after 3 months of exposure, the aquatic mosses were enriched in PAHs (enrichment factors are calculated through the ratio between PAH concentrations in the mosses after the 3 months of exposure and in the original control mosses) and allowed identifying illegal discharges into the streams. Aquatic mosses tend to accumulate pollutants in their tissues during the exposure period and if any discharge is made during this period, mosses will reflect this contamination signal.

In terrestrial environments, lichen transplants have also been used to monitor PAHs; Guidotti et al (2009), using transplants of the lichen *Pseudevernia furfuracea* have shown that 3 months of exposure are enough for the lichens to be enriched more than 90% . On the other hand, 2 months of exposure wasn't enough for the lichen *R. canariensis* to be enriched in PCDD/Fs when transplanted from a control site to a polluted site (enrichment was less than 10%) (Figure 3); only after 12 months of exposure, lichens became enriched more than 40%. The exposure period will depend on the intensity of pollution existent in the site to be monitored.

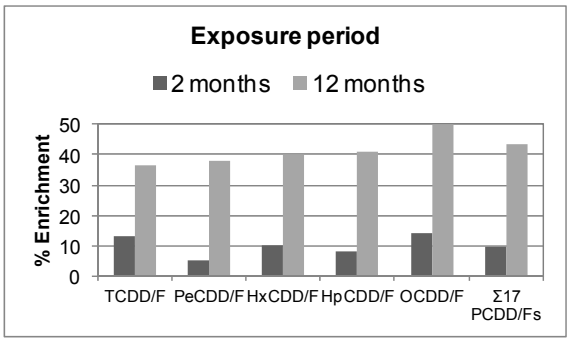

Figure 3. PCDD/F enrichment in lichens of the species *R. canariensis* transplanted from a control site to a polluted site for 2 (n=1) and 12 (n=1) months. Lichen transplants were prepared with 12 g of lichens inside a nylon bag, and suspended the bags in branches of pine trees.

When using biomonitor transplants to monitor POPs careful must be taken when selecting the exposure period. In the absence of time-studies designed to detect the minimal exposure period necessary to produce significant results, the relevance of the length of exposure remains to be investigated (Garty, 2001). POPs exist in the environment associated to other pollutants, namely metals; when biomonitors are transplanted, they will be subjected to the overall pollution existent at the monitoring site. In the case of lichens, relatively short exposure periods of 1-3 months are generally suggested since transplanted lichens may either lose some biomass or become saturated with elements, significantly altering their surface structure and physiological performance (Mikhailova, 2001). Lichens are known for their ability to accumulate airborne substances to concentrations far greater than those in atmosphere. Accumulation at ion exchange sites and especially particulate trapping are two well

documented mechanisms by which elevated levels of elements are achieved in lichens (Nieboer et al. 1978). However, elements present in trapped particulates may be dissolved in water or solubilized by the lichen to some degree, and metal ions released by this process may have several fates, e.g. become potentially capable of occupying cation binding sites in the cell wall or be taken up intracellularly (Kershaw, 1985). Nevertheless, the different metabolic fates of the various metals suggest that lichens may selectively accumulate those elements that remain extracellular, whereas those elements which enter into the cell may be metabolized and eliminated or may lead to death of the lichen (Kershaw, 1985). The mechanisms by which lichens accumulate POPs and the physiological impact of these pollutants on lichens are not understood yet, however it's well known that POPs exist in association with other pollutants which may lead to lichen death and compromise transplant results.

One of the most used methods to assess the physiological status of lichens and bryophytes is chlorophyll fluorescence, which is a non-destructive procedure to measure changes associated with photosystem II due to gaseous pollutants and heavy metals (Maxwell and Johnson, 2000). Using this method it's possible to evaluate how long can biomonitor transplants be exposed without being physiological affected by pollution. For that, measurements should be performed in biomonitors before and after exposure.

The use of transplanted material in study areas where natural/native lichens or mosses can't be found is a useful tool in pollution monitoring studies. With this work it was shown that when transplanting lichens or aquatic mosses, the exposure period should be selected depending on the intensity of pollution of the area to be monitored. After transplanting aquatic mosses from a natural stream to urban/industrial streams, 3 months of exposure were enough for the mosses to become enriched in PAHs and to identify different pollutant sources. However, when transplanting lichens from a natural/less contaminated area to a polluted one, only after 12 months of exposure, the lichens became enriched in PCDD/Fs. The transplanting period should therefore be always evaluated before a monitoring survey.

5.7. Environmental biomonitors as a tool in public health studies

Human exposure to POPs can occur by various pathways, notably inhalation of air and ressuspended soil particles, ingestion of food (which may include maternal milk), water and soil particles (relevant in the case of children), and dermal contact to soil and water (USEPA, 1998). To assess human exposure, usually concentrations of POPs deposited in air, soil, water, etc. are estimated based on emission data from known pollution sources. However, the uncertainty associated with the places where pollutants are predicted to deposit and where they are actually being deposited is very high, increasing also the uncertainty of the exposure data. Environmental observations for regulatory purposes are performed at air quality monitoring stations, using active samplers to capture POPs (PAHs, PCDD/Fs) retained in the particulate- and vapour-phases of atmosphere. These stations normally only few in space, tend to be located at specific sites selected for their expected high or low concentrations and are often placed at a much higher altitude than the human breathing zone (EHC 27, 1983). Moreover, the measurements reflect a short-time indicator that varies in time and which does not reflect the levels populations are exposed in space and in the long-term. In order to perform reliable epidemiological studies, environmental observations must be made in a way that reflects as closely as possible the exposure of the population under observation. The use of environmental biomonitors to assess levels of pollutants populations are exposed to allows obtaining information with a high spatial resolution. Lichens are long-lived biomonitors, and thus they are long-term integrators of atmospheric pollutant deposition. This feature is of crucial importance for evaluating human exposure to POPs; time integration of these compounds allows relating low levels of pollutants with long-term chronic effects on health, as shown in chapter 4.1. As demonstrated in chapters 4.1 and 4.2, at a regional scale, the use of environmental biomonitors will allow getting a real picture of pollutant deposition with enough spatial resolution to assess which populations should be considered as control and which ones should be considered exposed. Another option is to use biomonitors to estimate human exposure through inhalation (chapter 4.2). This can be done calibrating POPs in biomonitors against POPs in air, using the method described in chapter 2.4. Translating concentrations in biomonitors into the equivalent ones for air, it's possible to obtain measurements for POPs in air with a high spatial resolution, and thus estimate the daily dose of POPs humans are exposed to through inhalation (chapter 4.2). The use of lichens as estimators of air concentrations to assess

human exposure to POPs was shown in chapter 4.2, where lichens were used to estimate the intake of PAHs through inhalation of a population living close to a petrochemical region. In chapter 4.1, lichens were used as estimators of atmospheric PCDD/Fs at a regional scale, allowing assessing control and exposed populations for further development of health studies.

In this thesis it was shown how environmental biomonitors can be used to complement human health studies. The use of environmental biomonitors allows obtaining POP deposition data with high spatial resolution, allowing to assess which populations should be considered as control and which ones should be considered exposed. Translating concentrations in biomonitors into the equivalent ones for air, it was possible to obtain measurements for POPs in air with a high spatial resolution, and thus estimate the daily dose of POPs humans are exposed to through inhalation.

5.8. Guidelines: a perspective

For setting up a biomonitoring program using lichens (in terrestrial environments) or aquatic mosses (in aquatic environments) to assess environmental pollution by POPs, the most important thing to be considered at an early stage is to design the program (Figure 4). For that, the first thing that should be done is to clearly define which the main objective is; objectives can vary, from tracking pollution sources in the study area to assessing the impact of a given known pollution source, etc. The second step is to define the study area where the program will be implemented. These first steps will be useful to design the sampling grid and to decide which biomonitor to use. If the main aim is to assess the spatial impact of POPs emitted by a known industry, the sampling grid should be denser close to the industry and less dense as the distance increases. In this case, it's important to find specific indicators (which may be ratios between elements) of the known industry, so that it can be possible to distinguish it from other pollution sources.

The next step to be taken should be the collection of background information for the study area; this may vary accordingly to the aim of the work, but mostly it should include details regarding:

a) Sources and emissions - within cities sources include vehicles, industries, domestic etc., whereas in industrial areas, information should be obtained on the type of industries including their number, fuel used, composition of fuel, pollutants emitted etc. Information on number and distribution of sources should be collected. This information will help in identifying which pollutants can be expected in an area and thus should be measured.

b) Land use pattern – data on land use pattern should be acquired, as land use influences pollutants intercepted by biomonitors. For example, biomonitors collected in open land areas are most likely to show greatest concentrations of pollutants associated with soil particulates, whereas biomonitors collected in forest areas are most likely to show lowest concentrations of these pollutants.

c) Meteorological information – data regarding temperature and rainfall events, and also relative humidity, wind speed and direction, should be collected. Predominant wind direction plays an important role in determining dispersion of pollutants from emission sources and it will be useful for tracking pollution sources. Temperature and rainfall events (dry and wet depositions) are important parameters for POP deposition and their accumulation by biomonitors.

d) Topographical information - local winds and stability conditions are affected by topography. In river valleys there is increased tendency of developing inversions. More number of sampling sites should be located in areas where spatial variations in concentrations are expected to be large. Mountains, hills, water bodies also affect dispersion of pollutants and thus they shouldn't be neglected when designing the sampling grid.

e) Previous air/soil/water quality information - previous information collected for air, soil and water can serve as a basis for selecting areas where biomonitoring should be conducted. Previous studies can be used to estimate the magnitude of the problem.

f) Demography and population growth – areas where population density is high are expected to have greatest traffic flux and a greater input of pollutants in the environment due to a set of urban activities. Information on age and socio-economic status of population is also important for making a decision on an environmental biomonitoring survey for health purposes. Specific location of sampling sites will help in

finding exposure levels to population which can be used further in epidemiological studies to evaluate health effects of air pollutants.

g) Health and epidemiological studies (when the aim is related to health studies) - these will help to identify particular health impacts resulting from population exposure to air pollutants.

Once the background information is collected, the forth step is to design the biomonitor sampling grid. For that, and taking into account the objective of the study and the study area, the first thing is to confirm if biomonitors are existent and if the same species is present all over the area. If the area is devoid of biomonitors, the transplant technique should be used; for that, it will be needed to assess which is the best exposure period, i.e. time needed for the biomonitor to be enriched in the pollutant to be measured without being physiologically damaged (assessed through vitality measurements, such as chlorophyll fluorescence). If it's not possible to find the same biomonitor species all over the study area, a calibration between different species should be performed so that more than one species can be used in the same survey; for that, both species should be collected at the same sampling sites (covering different land uses with different intensities of pollution) and at the same time; calibration should be repeated during each spatial sampling campaign.

The fifth step corresponds to the spatial sampling campaign. Biomonitors should be collected when possible from 5 to 10 substrates (phorophytes) at each sampling site, at a height more than 1.5 m from the soil to minimize the influence of soil ressuspension. Collection should be performed during the shortest possible period, so that sampling is carried out under constant weather. These will minimize sampling variability and will make samples comparable between sites. After sampling, samples should be placed inside dark glass bottles and keep refrigerated until chemical analysis of POPs (sixth step).

Finally, the seventh step corresponds to the data analysis; results should be analysed accordingly to the main aims of the work. Data statistical treatment involving POP concentrations, POP profiles, ratios between POPs and different elements can be considered, as well as the use of Geostatistics to spatially build deposition models for POPs.

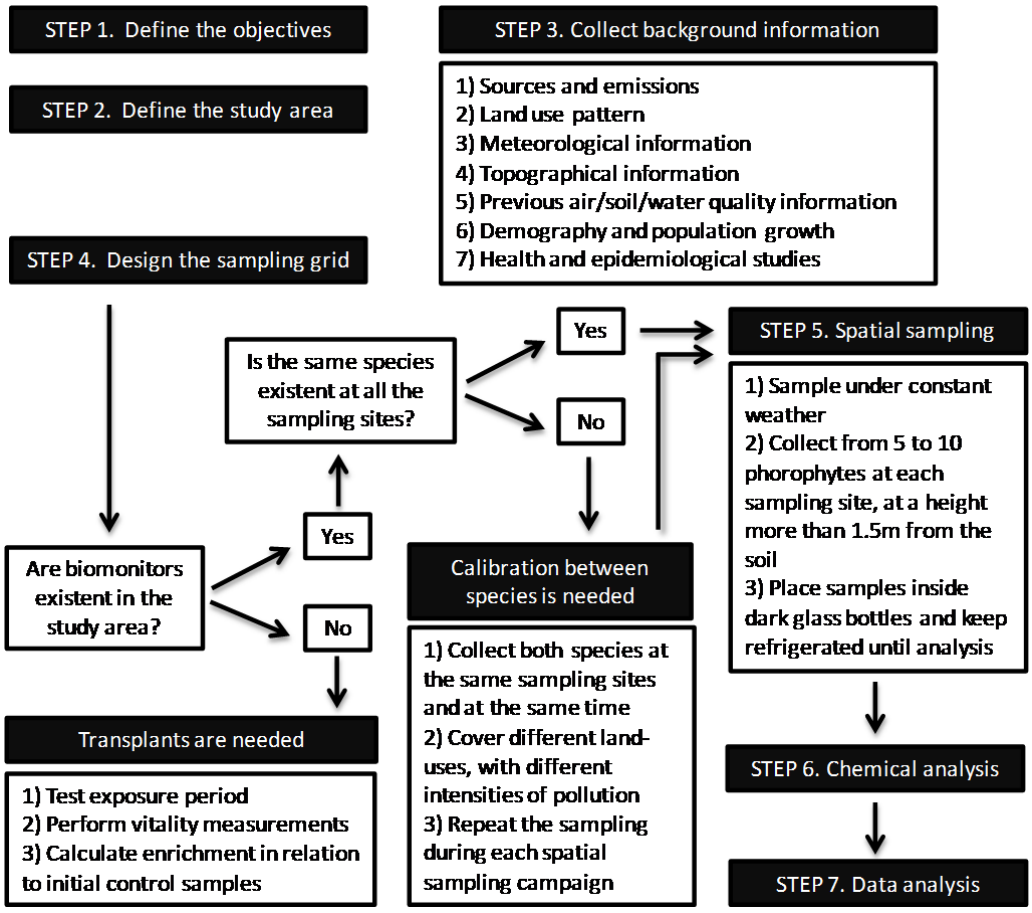

Figure 4. Program scheme for the use of biomonitors to assess POP deposition.

5.9. Final remarks

The aim of this thesis was to develop a technology for biomonitoring persistent organic pollutants (POPs) and for evaluating their impact on ecosystem and human health. As lichens have been the most used biomonitors in terrestrial environments to achieve pollution, in this thesis the main focus was given to these organisms. In aquatic environments, the focus was given to aquatic mosses.

In this work it was shown that fruticose lichens tend to accumulate greater concentrations of POPs than foliose ones, probably due to the highest surface/volume ratio. When comparing POP profiles, it was found that most species show similar profiles, with high contributions of lightest compounds (low molecular PAHs and less chlorinated PCDD/Fs), except the foliose lichen *X. parietina*, with a high longevity, which

has shown an influence of POP soil ressuspension. Even though POP profiles between *X. parietina* and *R. canariensis* were different, it was possible to establish a calibration between POP concentrations, allowing using both species in the same biomonitoring study if necessary. Substrate has shown to not be a critical factor influencing POP interception and accumulation by lichens.

It was also shown that concentrations of PCDD/Fs in lichens tend to decrease after a wet deposition period, being this decrease greatest for the highest molecular weight compounds (the most chlorinated PCDD/Fs, such as OCDD), meaning that probably these compounds are mostly retained at lichens' surface. Regarding temperature, it was shown that as temperature increases, PAH concentrations in lichens decrease, which is in accordance with what happens with PAHs in air.

Lichens have shown to be reflecting POPs existent in the air, either in the vapour- and particulate-phase. A calibration between lichens and air was possible, enabling to translate concentrations in lichens into equivalent concentrations for air, and thus into regulatory values. It was also shown that a calibration between lichens and soil is possible for the greatest concentrations.

With this work it was demonstrated the viability of using biomonitors, lichens in terrestrial environments and bryophytes in aquatic environments, to track POP pollution sources. For that, the analysis of POP profiles, land-use, and other pollutants (such as heavy metals) has shown to play an important role. The range of dispersion and deposition of a compound has shown to be dependent on its size. High molecular weight compounds tend to deposit close to emission sources, whereas low molecular weight ones tend to be ubiquitous and long-range transported.

The use of transplanted material in study areas where natural/native lichens or mosses can't be found is a useful tool in pollution monitoring studies. It was shown in this work that when transplanting lichens or aquatic mosses, the exposure period should be selected depending on the intensity of pollution of the area to be monitored. After transplanting aquatic mosses from a natural stream to urban/industrial streams, 3 months of exposure were enough for the mosses to become enriched in PAHs and to identify different pollutant sources. However, when transplanting lichens from a natural/less contaminated area to a polluted one, only after 12 months of exposure, the

lichens became enriched in PCDD/Fs. The transplanting period should therefore be always evaluated before a monitoring survey.

In this thesis it was shown how environmental biomonitors can be used to complement human health studies. The use of environmental biomonitors allows obtaining POP deposition data with high spatial resolution, allowing to assess which populations should be considered as control and which ones should be considered exposed. Translating concentrations in biomonitors into the equivalent ones for air, it was possible to obtain measurements for POPs in air with a high spatial resolution, and thus estimate the daily dose of POPs humans are exposed to through inhalation.

Finally, the integration of the information obtained in this thesis allowed designing a proposal of guidelines to be used when using environmental biomonitors to assess POP pollution.

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